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LEVELS OF YCG1 LIMIT CONDENSIN FUNCTION DURING THE CELL CYCLE

A Dissertation Presented

By

TYLER W. DOUGHTY

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 10th, 2016

CANCER BIOLOGY

LEVELS OF YCG1 LIMIT CONDENSIN FUNCTION DURING THE CELL CYCLE

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By
TYLER W. DOUGHTY

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ABSTRACT

For nearly five decades, the simple eukaryote *Saccharomyces cerevisiae* has been used as a model for understanding the eukaryotic cell cycle. One vein of this research has focused on understanding how chromosome structure is regulated in relation to the cell cycle. This work characterizes a new mechanism that modulates the chromatin organizing condensin complex, in hopes of furthering the understanding of chromosome structure regulation in eukaryotes.

During mitosis, chromosomes are condensed to facilitate their segregation through a process mediated by the condensin complex. Upon interphase onset, condensation is reversed, allowing for efficient transcription and replication of chromosomes. This work demonstrates that Ycg1, the Cap-G subunit of budding yeast condensin, is cell-cycle regulated with levels peaking in mitosis and decreasing as cells enter G1 phase. The cyclical expression of Ycg1 is unique amongst condensin subunits, and is established by a combination of cell cycle-regulated transcription and constitutive proteasomal degradation. Interestingly, when cyclical expression of Ycg1 is disrupted, condensin formation and chromosome association increases, and cells exhibit a delay in cell-cycle entry. These results demonstrate that Ycg1 levels limit condensin function, and suggest that regulating the expression of an individual condensin subunit helps to coordinate chromosome conformation with the cell cycle. These data, along with recent corroborating results in *Drosophila melanogaster* suggest that condensin

regulation through limiting the expression of a single condensin subunit may be broadly conserved amongst eukaryotes.

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PREFACE

Heather Arsenault performed the experiments in Figure 2.1C, Figure 2.6B, and Figure 2.8. In addition HA was strain generation in Chapter II.

Jennifer Benanti performed the experiments in Figure 2.1B, Figure 2.9, and Figure 2.12. In addition JB was instrumental in designing experiments and preparing the manuscript below.

Tyler Doughty performed and analyzed the remaining experiments, and was involved in experimental design and manuscript preparation.

Chapter II was taken almost verbatim from:

Doughty TW, Arsenault HE, Benanti JA. Levels of Ycg1 Limit Condensin Function During the Cell Cycle. PLoS Genetics, 2016

CHAPTER I

Introduction

Proliferation of eukaryotic cells depends on protection, transcription, replication, and segregation of Deoxyribonucleic Acid (DNA) polymers, which store genetic information. In order to accomplish these aims, cells progress through a unidirectional series of tightly regulated events, which together are known as the cell cycle. The cell cycle is divided into four phases: Growth Phase 1 (G1), DNA Synthesis Phase (S), Growth Phase 2 (G2), and Mitosis (M). Each of these phases represents a particular agenda; such as transcription/translation of necessary components for replication or cell division (G1 or G2 respectively), synthesis of a second copy of the genome (S), or DNA segregation and cell division (M) (Fig 1.1). Progression through each event in the cell cycle ensures that the two cells which emerge at the completion of mitosis contain a complete copy of the genetic material and are competent for subsequent proliferation.

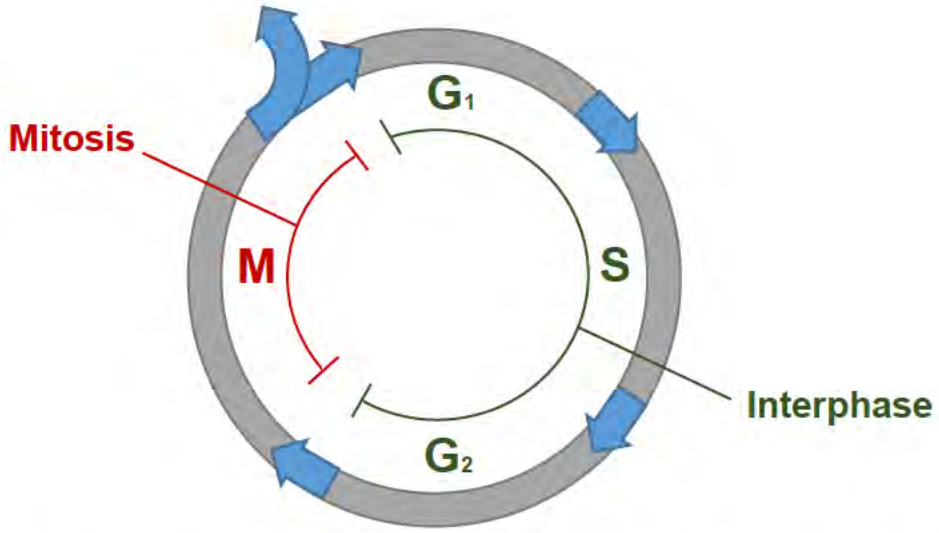


Figure 1.1: The Eukaryotic Cell Cycle. A diagram representing the phases of the cell cycle. Interphase is shown in green, with G1, S, and G2 sub-phases, while mitosis is shown in red. The cell cycle moves unidirectionally (blue arrows), and culminates in two daughter cells (forked blue arrow).

In eukaryotes, genetic information resides within the nucleus in the form of chromosomes, or large chains of DNA associated with proteins and RNA. This protein/DNA/RNA conglomerate structures compacts DNA to ~80-fold less volume than in its native state (Guacci 1994). This compact structure is highly dynamic, with chromosomes expanding or contracting during various cellular processes (Ehrenhofer-Murray 2004). The most notable amongst these changes in chromosome volume occurs during mitosis, when chromosomes are condensed by a mitotic proteins, resulting in an additional 2 to 10-fold decrease in volume (Guacci 1994, Koshland 1996). After the completion of mitosis, interphase (or

between phase) begins, and the protein component of chromatin changes, causing chromosomes to decondense from their mitotic form (Koshland 1996). (Fig 1.2)

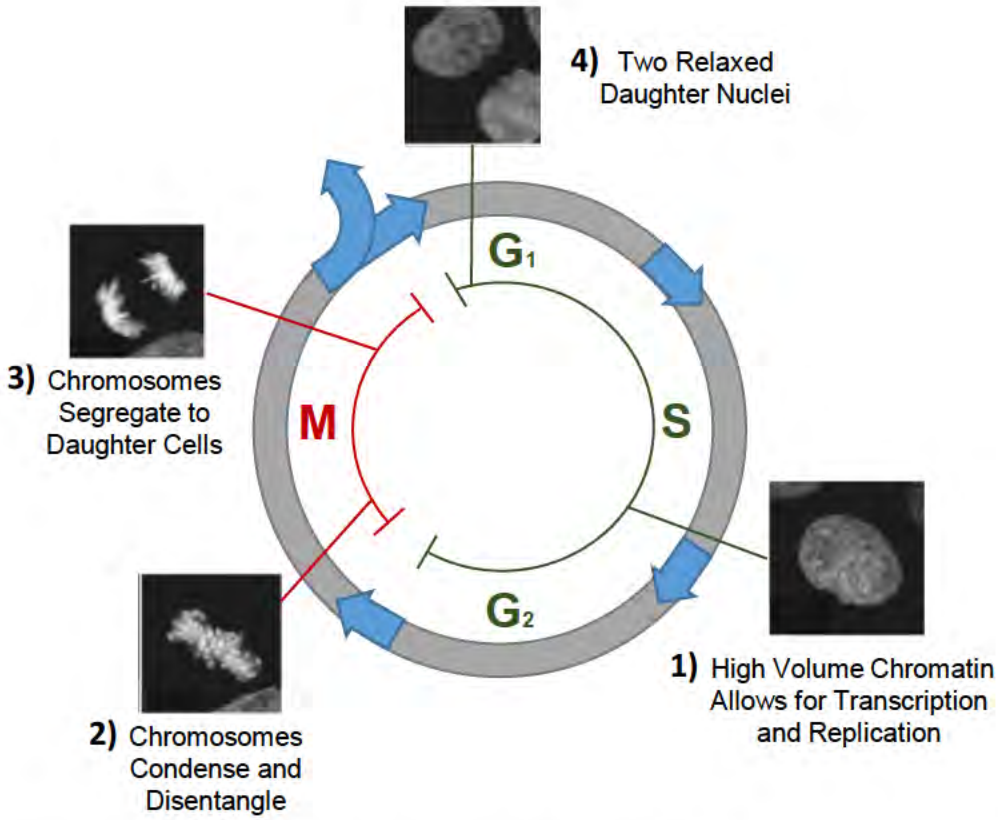


Figure 1.2: Chromatin States of the Eukaryotic Cell Cycle. A diagram representing the phases of the cell cycle and the corresponding chromatin conformation. 1) Chromosomes are highly volumous and accessible for transcription and replication during interphase (green), and 2 and 3) condense for mitotic segregation (red). 4) After segregation, two daughter cells emerge and chromatin relaxes to fill the nucleus. Microscopy images adapted from Hirota et al., 2004. Nuclear Chromatin is visualized by H2B-GFP in live human cell lines.

As alluded to in the previous paragraph, specific mixtures of proteins convert chromosomes between their interphase and mitotic states, but for what purpose? Further investigation into this question revealed that condensation during mitosis reduces chromosomal volume and individualizes chromosomes, so that they can be segregated during cell division (Koshland 1996, Nasmyth 2002). After segregation, interphase begins and chromosomal volume increases, allowing access for factors that execute the instructions coded in DNA. Later in interphase, chromosomes become topologically entrapped (tangled) as a byproduct of replication (Hirano 2016). These tangles are resolved in late interphase and early mitosis to facilitate chromosome segregation. Thus, the fate of the eukaryotic cell cycle is entwined with the DNA that it exists to propagate.

The Condensin Complex is a Conserved Modulator of Chromosomal Structure

The condensin complex is a key modulator of chromosomal volume that is conserved and essential throughout eukaryotes (Hirano 2012) (Figure 1.3). Condensin regulates chromosomal volume by interacting with multiple strands of DNA on the same chromosome, and via a largely undefined ATP driven mechanism, induces positive supercoils into DNA (Kimura 1997, St. Pierre 2009, Piazza 2014). This positive supercoiling activity of condensin is present at a basal level throughout the cell cycle, and is hyperactivated during mitosis (Takemoto 2004, St. Pierre 2009). Mitotic hyperactivation of condensin compacts the genome and helps resolve topological entanglements (Hirano 2012). Condensation is

reversed after the completion of mitosis, via an undefined mechanism, which likely involves returning condensin activity to a basal level, thus allowing chromosomes to relax during interphase.

Other Notable Factors Which Contribute to Mitotic Chromosome Structure

Condensin is one of many factors necessary for mitotic chromosome structure and mitotic segregation. In this section, we introduce additional factors that impact chromosome structure, and explain how these factors, and condensin, work together to drive mitotic chromosome condensation/segregation.

Topoisomerases are enzymes which transiently break DNA to alter its writhe (supercoiling) or resolve entanglements (Wang 2002). Topoisomerases are important for resolving supercoils associated with replication and transcription, and play essential roles in chromosome condensation (Wang 2002). Topoisomerase I family enzymes (topo I) cut a single strand of a DNA double-helix and alter its writhe, by relaxing negative (Topo IA) or positive and negative (Topo IB) supercoils (Tsao 1989, Yang 1989). Condensin and either Topo IA or IB are necessary and sufficient to induce positive supercoils in plasmids *in vitro* in an ATP dependent manner (Kimura 1997). This is due to condensin's ability to positively supercoil DNA, and protect these positive supercoils from relaxation, while allowing negative supercoils to be relaxed by topoisomerase (Kimura 1997). The interplay between Topo I and condensin on mitotic chromosomes *in vivo* is at present uncharacterized.

Topoisomerase II (topo II) disentangles chromosomes by cutting complementary strands of double stranded DNA, which allows another double-stranded DNA to pass through (Wang 2002). This activity is essential for mitotic segregation, since interchromosomal links impede separation of chromosomes from one another. The positive supercoils induced by condensin have been shown to recruit topo II *in vitro*, ultimately leading to disentanglement of linked plasmids (Wang 2002, Baxter 2011). This appears to also be the case *in vivo,* where condensin activity is necessary to recruit Topo II, which in turn is required to resolve interchromosomal overlaps (Baxter 2011, Leonard 2015). The final result of these processes is compact and individualized chromosomes, which are competent for mitotic segregation.

Cohesin is a four subunit protein complex, which is structurally similar to condensin, and is conserved throughout eukaryotes. The cohesin complex is essential for pairing sister chromatids after replication. This pairing is maintained until anaphase, when cleavage of cohesin releases sister chromatids for segregation (Nasmyth 2009). Cohesin pairs sister homologs by encircling dsDNA topologically (Ivanov 2005), though the mechanism is still unclear as to how this leads to sister chromatid pairing (Nasmyth 2009). Nonetheless, pairing ensures equal segregation of chromosomes during mitosis by keeping sister chromatids together until spindle pole attachment (Guacci 1997, Uhlmann 2000, Peters 2008). In addition to homolog pairing, cohesin participates in mitotic chromosome structure in budding yeast (Guacci 1997). This is potentially due to

intrachromosomal interactions, which might account for the observed cohesin-dependent changes in intrachromosomal distances (Guacci 1997, Lopez-Serra 2013). These functions, along with the aforementioned activities of condensin and topoisomerase, ensure proper condensation, disentanglement, and biorientation of chromosomes during mitosis.

Condensin Discovery and Characterization

This section seeks to introduce how condensin has been characterized experimentally, with some emphasis on tools and observations from the model organism budding yeast. This information is the foundation which has guided the hypotheses and experimental approaches in Chapter II.

Condensin was initially discovered as a factor necessary for condensation in *Xenopus laevis* sperm chromatin (Hirano 1997). In these experiments, mitotic extracts were insufficient to condense sperm chromatin after depletion of condensin by ~95% (Hirano 1997). Further, depletion of non-SMC subunits made extracts incompetent for mitotic condensation, suggesting that the condensin SMC and non-SMC subunits are necessary for this phenotype (condensin subunit composition shown in Figure 1.3). Soon after this initial characterization, the condensin complex was identified in budding yeast, flies, mice, and humans (Hirano 1997, Sutani 1999, Schmiesing 2000, Freeman 2000).

Immunoprecipitation followed by silver staining in budding yeast showed that condensin subunits participate in the complex at 1:1:1:1:1 ratios (Freeman

2000); this ratio is likely the same in other eukaryotes (Hirano 2012). Temperature sensitive alleles have been established for each condensin subunit in budding yeast (Freeman 2000, Ouspenski 2000, Bhalla 2002, Lavoie 2002). In this system, condensin inactivation during mitosis increases the occurrence of lagging chromosomes, causes decondensation of chromosomes, and changes the morphology of the ribosomal DNA locus on Chromosome XII (Freeman 2000). Condensin inactivation also increases the distance between LacO arrays on the same chromosome arm (monitored by LacI-GFP) (D'Ambrosio 2008), suggesting that it contracts chromosome arms toward the centromere. Chromatin-IP identified mitotic condensin enrichment sites including centromeres, ribosomal DNA, and telomeres (Wang 2005, D'Ambrosio 2008, Verzijlbergen 2014, Leonard 2015). In addition, condensin activation has also been shown to be required for segregation during meiosis, by resolving topological links between chromosomes (Yu 2005), and aiding in coorientation during meiosis I (Brito 2010).

Chromatin-IP identified condensin association with tRNA genes, ribosomal protein genes, and ribosomal RNA genes in yeast during interphase (D'Ambrosio 2008, Leonard 2015). A similar interphase binding footprint including enhancers, active promoters, and tRNA genes was observed in *C.elegans* and mouse embryonic stem cells (Dowen 2013, Kranz 2013). Condensin inactivation during interphase de-clusters many of the 274 tRNA gene loci from the peri-nucleolus in budding yeast (monitored by FISH) (D'Ambrosio 2008, Haeusler 2008, Leonard 2015), suggesting that condensin clusters regions during interphase. Similarly,

Condensin inactivation in *Drosophila*, mouse embryonic stem cells, and transformed human cell lines disrupts nuclear morphology and causes chromatin to swell (Fazzio 2010, George 2014). However, condensin inactivation does not slow interphase progression in yeast (Chapter II) or HeLa cells (Ono 2013). Together, these data show that condensin associates with chromatin during interphase and impacts gene expression and chromosome structure, but is dispensable for interphase progression.

Overexpression of one condensin subunit (Brn1) in budding yeast is associated with G2 accumulation, while overexpression of another (Ycg1) causes increased metal resistance (Sopko 2006, Hwang 2009). The other condensin subunits have not exhibited measurable phenotypes upon overexpression. However, these results are from large scale screens, which are not exhaustive, and thus condensin subunit overexpression requires further study. Prior to the findings presented in Chapter II, neither subunit overexpression nor mutation has been shown to increase condensin levels or activity in budding yeast.

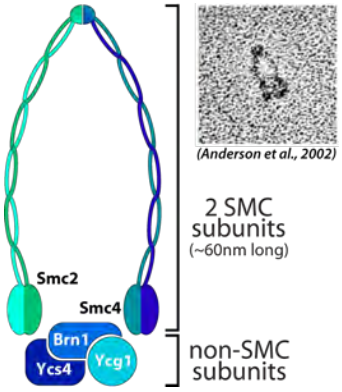


Figure 1.3: Cartoon of the Eukaryotic Condensin Complex. A cartoon of the eukaryotic condensin complex is shown. Condensin is a conserved and essential complex found throughout eukaryotes, which is required for mitotic chromosome condensation. Budding yeast subunit names are shown. An electron microscopic image is shown (top right). Originally from the D'Amours lab University of Montreal.

Subunits of the Eukaryotic Condensin Complex

Eukaryotic condensin complexes are constituted of five proteins; two SMC family subunits, two HEAT repeat subunits, and a kleisin subunit (Hirano 1997). In systems which employ two condensins, the SMC family subunits are shared between condensins I and II, while the non-SMC subunits are specialized for each complex (Hirano 2012) (Fig 1.4).

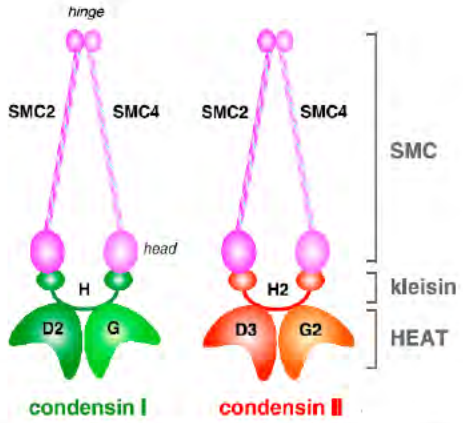


Figure 1.4: Condensin Complex Architecture. Condensin subunits Smc2, Smc4, and CapH form a closed loop that can encircle a strand of DNA. CapD2 and CapG contain HEAT repeats, and associate with CapH. Some eukaryotes encode a second condensin complex, Condensin II, which utilizes the same Smc subunits, but has a unique set of non-Smc subunits. Figure adapted from Hirano, 2012.

Structural Maintenance of Chromosomes (SMC) subunits are coiled-coil proteins which hinge in the center, coil along themselves, and culminate in an ATPase domain formed by their N and C-terminal ends (Strunnikov 1993). Together the two condensin SMC subunits form a heterodimeric ring-like structure, with hinges interacting on one side, and heads interacting on the other (Hirano 2012). ATP hydrolysis allows these subunit's heads to separate from one another and encircle a strand of DNA, which is entrapped by the kleisin subunit that caps the SMC ring (Akai 2014). In addition to capping the SMC ring, the kleisin subunit acts as a scaffold, as it is the only subunit which interacts with all other condensin subunits (Piazza 2014). The CapG and CapD condensin subunits interact with the kleisin subunit and extend outward with their HEAT repeat domains. These HEAT domains are orthologous to the protein-protein interaction domain found on

Huntingtin, **E**longation factor 3, PP2**A**, and **T**OR1 (Kobe 1999). However, unlike the aforementioned proteins, condensin HEAT-repeat subunits likely interact with DNA directly, and appear to initiate the condensin-DNA interaction *in vitro* and *in vivo* (Piazza 2014). Together, the subunits of condensin interact with two strands of DNA, thus bridging otherwise distant chromosomal regions (Fig 1.5).

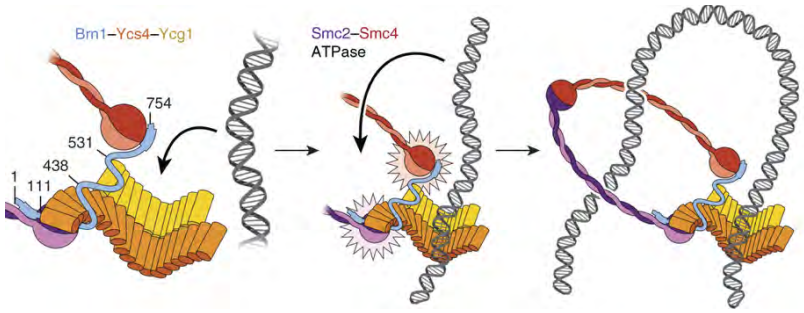


Figure 1.5: Condensin Interacts with Multiple Strands of DNA to Facilitate Clustering. A cartoon showing a hypothetical mechanism by which condensin interacts with DNA. *In vitro* data suggest that the HEAT repeat subunits interact with one strand of DNA, followed by ATP dependent opening of the SMC hinge, leading to entrapment of a second strand. Figure adapted from Piazza et al., 2014.

Budding Yeast as a Model for Characterizing Eukaryotic Condensin

Since condensin's initial discovery in *Xenopus laevis* (Hirano 1997), the complex has been found to be conserved in yeasts, flies, plants, mammals, and bacteria (Hirano 2012). However, condensin has diverged in these various organisms, existing in a simplified form in bacteria, as one complex in some eukaryotes (some yeasts), two complexes in others (flies, mice, and humans), and

in three forms in at least one case (*C. elegans*) (Hirano 1997, Sutani 1999, Schmiesing 2000, Freeman 2000).

In the previous sections, condensin's role in interphase clustering and decatenation, as well as mitotic condensation has been described. In mammals, these functions are divided between two condensin complexes, condensin I and condensin II (Hirano 2012). Condensin II is nuclear throughout the cell cycle, where it performs interphase functions and participates in mitotic condensation (Shintomi 2011). Condensin I is sequestered in the cytoplasm until nuclear envelope breakdown in early mitosis, when it accesses chromatin and aids in condensation (Shintomi 2011, Hirano 2016).

The functions of condensin I and II are performed by a single condensin complex in budding yeast (Freeman 2000, Lavoie 2002). Over the past two decades, budding yeast condensin has proven to be a reliable model for condensin function in eukaryotes. This research has found that yeast condensin exhibits the same positive supercoiling activity, in response to phosphorylation by many of the same kinases as multicellular eukaryotes (Hirano 1997, Sutani 1999, Schmiesing 2000, Freeman 2000, Lavoie 2004, St. Pierre 2009). In addition, the known biochemical activities of yeast condensin, and condensin I and II are the same; i.e. all condensins bind to chromatin and induce positive supercoils (Hirano 2016). Finally, condensins across eukaryotes participate in the same interphase organization and mitotic condensation of chromatin. Together, these similarities

suggest that yeast is a good model system for understanding eukaryotic condensin. Table 1.1 shows condensin subunit names in select eukaryotes.

Subunit	*Homo sapiens*	*Drosophila melano.*	*S. pombe*	*S. cerevisiae*
SMC2	*SMC2*	*SMC2*	*cut14*	*SMC2*
SMC4	*SMC4*	*SMC4*	*cut3*	*SMC4*
Kleisin	*NCAPH*	*Barren*	*cnd2*	*BRN1*
HEAT A	*NCAPG*	*CAP-G*	*cnd3*	*YCG1*
HEAT B	*NCAPD2*	*CAP-D2*	*cnd2*	*YCS4*
Kleisin II	*NCAPH2*	*CAP-H2*	-	-
HEAT A II	*NCAPG2*	*	-	-
HEAT B II	*NCAPD3*	*CAP-D3*	-	-

Table 1.1: Condensin Subunits in Select Eukaryotes: Condensin subunit gene names are shown for select eukaryotes. Note that *S.pombe* and *S.cerevisiae* do not encode condensin II subunit genes. *Drosophila melanogaster* HEAT A II subunit is denotes as an * because this subunit has not been identified.

Condensin Recruitment to DNA is Regulated Spatially and Temporally to Modulate Genome Organization

Condensin loading to many genomic loci requires chromatin associated receptors and/or post translational modification. This section summarizes the currently characterized loading factors, with a primary focus on the model system budding yeast. In budding yeast, condensin is loaded onto chromatin by several

independent mechanisms, which recruit condensin to specific regions during each cell cycle phase.

Shugoshin, or Sgo1 in yeast, is a centromere localized protein which is involved in sensing spindle tension (Vahan 2005). Recent findings show that shugoshin recruits condensin to centromeres and pericentric regions to facilitate chromosome biorientation during metaphase (Verzijlbergen 2014, Peplowska 2014). This recruitment is due to a physical interaction between condensin and Sgo1, that may require the phosphatase regulator Rts1 and/or monopolin (Burrack 2013, Peplowska 2014). Notably, Sgo1's centromeric localization begins in late S-phase, causing very high condensin loading at and around the centromeres at this time (Verzijlbergen 2014). This enrichment is maintained until the start of anaphase, when condensin moves to the chromosome arms. Disruption of condensin recruitment to centromeres causes failure to biorient chromosomes, thus decreasing the fidelity of mitosis (Verzijlbergen 2014, Peplowska 2014).

Fob1, or Fork Blocking less, is a protein which associates with the ribosomal DNA (rDNA) in budding yeast. The rDNA is a locus on Chromosome XII that encodes ~150 repeats of the ribosomal RNA subunit genes (Johzuka 2006). Fob1 sits at a single site on the rDNA, the RFB, or Replication Fork Blocking site, and ensures replication forks move unidirectionally through this locus to avoid collision with RNA Polymerase I (Johzuka 2006). Interestingly, Fob1 and monopolin subunits, Tof2, Csm1, and Lrs4, physically interact with and recruit condensin to RFB during anaphase (Johzuka 2006, Johzuka 2009). This recruitment is

extensive and vastly enriches the rDNA occupation of condensin, which is essential for disentanglement, condensation, and segregation of Chromosome XII (Johzuka 2009). After the completion of mitosis, some condensin remains at the rDNA, primarily to protect the transcriptionally inactive repeats from improper homologous recombination (Tsang 2007).

Aurora B kinase is a regulator of several mitotic processes, including the spindle checkpoint and chromosome segregation (Biggins 2001, Marko 2002). Recent work in fission yeast and human cell lines showed Aurora B phosphorylation of condensin facilitates condensin loading onto chromatin (Tada 2011). Aurora B accomplishes this by phosphorylating the kleisin subunit of condensin (CapH) and nucleosome subunits H2A and H2A.Z to facilitate nucleosome/condensin interaction (Tada 2011). Unlike Fob1 and Sgo1, which each recruit condensin to a specific locus, phosphorylation of CapH and H2A/H2A.Z facilitates condensin loading at loci throughout the genome (Tada 2011). Since Aurora B primarily resides/acts at the midzone during mitosis, it is thought to facilitate condensin loading at midzone proximal loci. Indeed, this is observed experimentally, as condensin associates with centromeres during metaphase, then leaves these regions (after they move away from the midzone) for chromosome arms which segregate last during mid-anaphase (Tada 2011, Afonso 2014).

Condensin loading is also impacted by TFIIIC, a complex which recruits RNA Polymerase III to genes, such as tRNA genes. Condensin physically interacts

with TFIIIC (Haeusler 2008), and is found to bind many tRNA genes in budding yeast, with a proclivity for these loci during interphase (D'Ambrosio 2008, Leonard 2015). Disruption of condensin function scatters the tRNA gene cluster, which is normally proximal to the nucleolus, and leads to loss of silencing in nearby Pol II genes (Haeusler 2008). In fission yeast, TBP or TATA binding protein is also important for the recruitment of condensin and gene clustering during interphase, though this has not been assessed in budding yeast (Iwasaki 2015). Interestingly, both TBP and TFIIIC clustering bring together loci scattered amongst multiple chromosomes (increasing interchromosomal associations) while mitotic chromosome condensation decreases interchromosomal associations and individualizes chromosomes. The factors that dictate whether condensin will exhibit intra or interchromosomal function are currently unknown.

Genome-wide condensin association suggests that other loci (non-TFIIIC/non-TBP loci) are enriched for condensin during interphase (Piazza 2014, Leonard 2015). Binding at these sites may be facilitated by condensin's intrinsic affinity for open DNA, which has been described in recent *in vitro* studies (Kimura 1997, St. Pierre 2009, Piazza 2014). Indeed, these *in vitro* data correlate well with condensin binding to nucleosome depleted regions genome wide (Piazza 2014, Leonard 2015), and suggest that condensin may enrich at some loci independent of *bona fide* loading factors. Recent data suggests that this "naked" DNA binding is mediated by the SAGA (histone acetyltransferase) complex in fission yeast,

which moves nucleosomes, creating nucleosome depleted regions, where condensin loads (Toselli-Mollereau 2016).

Together, these diverse loading mechanisms establish regulated condensin recruitment during the cell cycle. This spatial and temporal regulation of condensin localization helps focus condensin activity to the appropriate loci during each cell cycle phase. Section summarized in Table 1.2.

Chromatin Receptor	Region of Recruitment	Cell Cycle Position	References
Shugoshin	Centromere and Peri-centromere	Late S-phase to Mid Mitosis	Burrack 2013 Peplowska 2014 Verzijlbergen 2014*
Fob1	Ribosomal DNA	Anaphase	Johzuka 2006* Johzuka 2009*
Phospho-H2A/ Phospho-H2A.Z	Genome Wide	Mitosis	Tada 2011 Afonso 2014
TFIIIC	tRNA snoRNA	Interphase	Haeusler 2008* D'Ambrosio 2008*
TATA-Binding Protein	Active Genes	Interphase	Iwasaki 2015
Nucleosome Depleted DNA	Transcriptional Start sites	Interphase	Piazza 2014*

Table 1.2: Condensin Loading in Yeasts: Condensin loading receptors, their localization and the cell cycle timing of recruitment is shown. Research carried out in budding yeast is denoted by an (*).

Phosphorylation Regulates the Supercoiling Activity of Condensin

In addition to spatial and temporal regulation of condensin loading, condensin's enzymatic activity changes during the cell cycle in response to

phosphorylation. Phosphorylation impacts condensin activity by 1) increasing condensin's affinity for chromatin (discussed above), 2) activating supercoiling (mitotic kinases), or 3) blocking supercoiling (CKII kinase). In addition, condensin subunits are post-translationally modified by ubiquitination, SUMOylation, and acetylation (Peng 2003, Wohlschlegel 2004, Takahashi 2008, Choudhary 2009), but the consequence of these modifications is at present uncharacterized. Phospho-activation and phospho-repression are discussed in detail below.

In vitro supercoiling assays suggest that condensin exhibits a basal level of supercoiling activity during interphase and high levels of supercoiling activity when phosphorylated by mitotic kinases (Takemoto 2004, St. Pierre 2009). Notably, mitotic kinase phosphorylation of interphase condensin is sufficient to increase supercoiling activity *in vitro* (Kimura 1997, St. Pierre 2009). Changes in supercoiling activity might explain how the same complex clusters a small number of genomic loci during interphase (tRNA genes) (Haeusler 2008), and condenses entire chromosomes during mitosis (Lavoie 2004). Specifically, the aforementioned phosphorylation is associated with Polo kinase (Cdc5) and the mitotic form of Cyclin-dependent kinase (Cdk-Cyclin B) (Kimura 1997, Lavoie 2004, Abe 2011, Robellet 2015), though several other kinases phosphorylate condensin subunits for other purposes (Bazile 2010, Nguyen 2015, Robellet 2015). Cdc5 and Cdk-Cyclin B activate condensin during mitosis, but are extinguished in late mitosis/early G1. The inactivation of these kinases, along with phosphatase

activity (yet to be identified), may reset the complex to a basal phosphorylation state, causing decondensation upon completion of mitosis.

Conversely, phosphorylation by CKII has been shown to inhibit condensin supercoiling during interphase in human HeLa cells (Takemoto 2006). *In vitro*, this modification overrides activating marks and acts as a break for the complex, and, upon removal may contribute to switch-like activation during mitosis *in vivo* (Takemoto 2006). CKII consensus sites on condensin subunits are phosphorylated in budding yeast, but the consequence of phosphorylation at these sites are currently uncharacterized (Bazile 2010).

Protein Expression During the Cell Cycle – Cyclical Transcription

A novel mechanism which regulates the total amount of condensin during the cell cycle is described in Chapter II. This mechanism causes cyclical protein expression of a single condensin subunit. This section and the following section seek to examine the interplay between transcription and proteasomal targeting that causes cyclical expression of previously characterized proteins.

Early analysis of the transcriptome, or the entire mRNA content expressed in an organism, identified a large subset of genes which are cyclically transcribed (~15% in budding yeast) (Spellman 1998), i.e. their transcript levels oscillate in a manner which correlates with cell cycle position. Several of these cyclically transcribed genes code for cyclical proteins, which rise in expression level during specific phase(s) of the cell cycle and drop in others (Sullivan 2007). However, transcriptional silencing alone is not sufficient to significantly decrease protein

levels during the relatively short time spent in a given cell cycle phase. Thus, transcriptional changes are often paired with changes in protein turnover to rapidly impact protein levels. An example of cyclical expression is the eukaryotic cyclin-B protein, which is highly transcribed and stable during G2 and early mitosis, followed by transcriptional silencing and rapid protein turnover in late mitosis (Hagting 2002, Morgan 2007). This regulation allows cyclin-B, an integral player in mitotic progression, to be expressed at high levels for a brief window in mitosis, followed by very low expression during the following G1.

Protein Expression During the Cell Cycle – Protein Turnover

As alluded to above, protein turnover rates can be modulated during the cell cycle. Indeed, protein turnover is a tightly regulated process which is primarily facilitated by Ubiquitin-Proteasome System (UPS) mediated proteolysis in eukaryotes (Ciechanover 1980, Yen 2008). UPS mediated proteolysis is a multi-step process, where a protein of interest is specifically recognized, covalently poly-ubiquitinated, and is subsequently targeted for proteasomal degradation (Hochstrasser 1996, Hershko 1998). The initial step, protein recognition, requires a set of enzymes which covalently attach ubiquitin to themselves via a transesterification reaction (Hershko 1983, Hochstrasser 1996). The enzymes involved are E1, E2, and E3 ligases, with E3-ligases representing the most diverse class (Hershko 1998). In the second step, an E3-ligase specifically recognizes a unique region of a protein called a degron, and covalently attaches a ubiquitin moiety onto a Lysine residue on the target protein (Varshavsky 1991, Hochstrasser

1996, Ravid 2008). These ubiquitin proteins are often linked to multiple Lysine residues on the target protein, and onto one another, forming long chains (Chau 1989).

Finally, substrates with critically long ubiquitin chains are recognized and targeted to the proteasome, a large multi-subunit protease, for destruction (Chau 1989). Notably, some degron-E3 ligase interactions are cell cycle specific, leading to protein turnover in a specific phase, while others occur constitutively. (Fig 1.6)

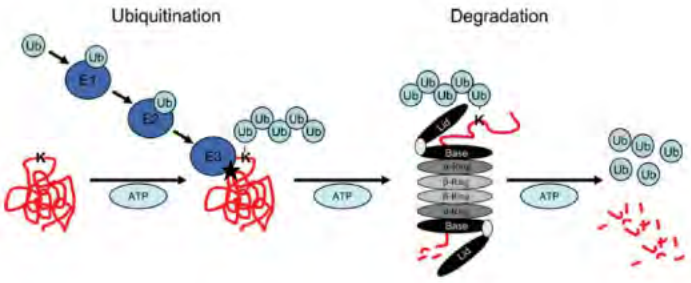


Figure 1.6: Protein Degradation by the Ubiquitin Proteasome System. A protein of interest (red squiggle) is targeted for ubiquitin-mediated degradation via specific recognition of its degron region (star) by an E3-ligase. Upon recognition, the E3 ligase transfers a ubiquitin to a degron proximal Lysine (K) residue on the target protein or onto an already present ubiquitin. Ubiquitin chain formation signals recruitment of the protein of interest to the proteasome, where it is degraded. Adapted from Carrier 2009.

These two parameters, transcription and turnover, could lead to cyclical expression in three ways; 1) transcription and turnover rates depend on cell cycle position (example cyclin-B), 2) transcription is constitutive and turnover rates vary with the cell cycle, or 3) transcription varies and turnover is constitutive (described herein). In any case, cyclical expression is energy intensive, due to constant

construction and destruction of protein. However, for many proteins, cyclical expression is necessary to ensure that cell cycle phase specific functions occur only during the appropriate phase.

The G1/S Restriction Point or "START"

Eukaryotic G1/S cell cycle progression has been studied extensively in budding yeast and mammalian cultured cells. In both cases, commitment to the cell cycle happens in a switch-like manner, called the restriction point in mammals or START in budding yeast (Costanzo 2004, de Bruin 2004). This transition from G1 to S-phase is highly regulated to ensure that a cell does not commit to the cell cycle until the proper conditions permit. Below a brief description of the START mechanism in budding yeast is provided in order to introduce the general concept of cell cycle control by transcription and proteasomal function. In addition, the START mechanism is described to prime the reader for data presented in Chapter II.

During the early cell cycle, intracellular signaling pathways drive G1/S transcription and cellular progression through START (Costanzo 2004, de Bruin 2004). In budding yeast, this regulation relies on SBF and MBF transcription factors, and their repressor Whi5 (Bertoli 2013). SBF and MBF bind promoters of genes important for G1/S functions, but do not actively facilitate transcription in the presence of Whi5 (Costanzo 2004, de Bruin 2004). During progression through G1, Cdk-Cln3 phosphorylates Whi5, leading to transcriptional activation of some

early G1/S genes, including *CLN1* and *CLN2* (Costanzo 2004, de Bruin 2004). The small amount of Cln1 and Cln2 protein that results from this associates with Cdk, and leads to feedback inhibition of Whi5, eventually derepressing hundreds of genes (Bertoli 2013). Many of the gene products expressed during START function in the subsequent cell cycle phase or act as regulators which feedback to end the START wave of gene expression (Bertoli 2013).

The cyclical expression of the master regulators of START and other cell cycle specific proteins expressed downstream of these regulators are paramount in establishing discreet cell cycle phases. An example is Cln2, which is expressed at a high level during G1/S, but low levels in other stages of the cell cycle (Schneider 1998). This cyclical expression is the result of repressed transcription outside of G1/S, and constant proteasomal targeting (Schneider 1998). This mechanism ensures that Cln2 drives START, via Whi5 derepression specifically during G1/S.

Internal and external inputs can act as a brake to delay cell cycle commitment. In budding yeast, the inputs which can halt G1/S progression include nutrient availability, mating factors, and cell size (Jorgensen 2004, Benanti 2012, Shi 2013). Many of these brakes inhibit G1/S progression by inhibiting the transcription, translation, function, nuclear localization or (possibly) stability of Cln3 (Barbet 1996, Hall 1998, Gari 2001, Landry 2012, Shi 2013). In turn, the lack of Cdk-Cln3 function halts *CLN1* and *CLN2* transcription and thereby, the entire G1 to S phase transcriptional wave. Progression through START preceeds bud

formation in budding yeast, which allows progression through START to be monitored visually.

Altered Condensin Levels in Cancer

This work seeks to characterize changes in condensin levels in budding yeast. These findings may inform a deeper understanding of condensin regulation, which may be useful for understanding recent observations in the field of Cancer Biology.

Perturbations in condensin subunit levels have been observed in cancerous cells, though condensin's role in proliferation appears to vary in different cell lines. One example of altered condensin expression comes from Wnt/β-catenin/Tcf4 colorectal carcinomas, which show increased condensin subunit expression (Davalos 2012). Knockdown of *SMC2* in these carcinomas, to levels similar to primary cells, caused mitotic cell death and decreased proliferation, likely due to mitotic catastrophe (Davalos 2012). A similar finding showed increased expression of SMC2 in neuroblastomas, with subsequent knockdown leading to reduced proliferation. In this case, MYCN drives an increase in SMC2 expression, which facilitates SMC2 dependent DNA damage response gene expression during interphase (Murakami-Tonami 2014). These findings were surprising, since condensin knockdown in HeLa cells slowed mitotic progression, but only marginally, suggesting that relatively small amounts of condensin are sufficient for survival (Hirota 2004, Hirano 2012). The condensin-cancer link is further

complicated by observations that condensin subunits are decreased in pyothorax-associated lymphoma (Ham 2007), and that subunit overexpression induces differentiation in erythroleukemia (Xu 2006).

This complicated mixture of condensin dependencies in various cancers appears counterintuitive. In some cases, increased condensin appears to aid in proliferation, due to a role in managing the DNA damage or chromosome instability inherent to transformed cells (Davalos 2012, Murakami-Tonami 2014). In other cases, decreased condensin may aid proliferation by increasing genetic mutation rates (Ham 2007). Though these findings are intriguing, it is difficult to weigh their implications due to current holes in the understanding of condensin biology. While condensin levels and their implications on proliferation have been studied extensively via knockdown and inactivation in various model systems, the effects of increasing condensin levels have remained uncharacterized until recently (Buster 2013, Chapter II).

Additionally, it is worth noting that in many of the above cases, a condensin subunit, or a few subunits show increased expression, but others remain unchanged or unassessed (Davalos 2012, Murakami-Tonami 2014). These data are curious, since condensin subunits are not known to function independent of the condensin complex. In Chapter II, we characterize a mechanism by which *bona fide* condensin levels increase upon changes in expression of a single complex subunit in budding yeast. Additionally, we characterize the consequences of increased condensin levels on proliferation. These findings may help elucidate

mechanisms which lead to increased condensin levels in cancer cells, and may further the understanding of condensin's role in proliferation in these malignancies.

Ycg1 Expression Limits Condensin Formation and Association with Chromatin

This work describes the cyclical expression of the CapG condensin subunit in budding yeast and its effect on condensin function. It has previously been shown that all five subunits are necessary for condensin activity; i.e. the least abundant subunit of the complex determines how much condensin can form (Lavoie 2002, Lavoie 2004, D'Ambrosio 2008). Our observations suggest that in budding yeast, condensin is limited by the expression of the yeast CapG subunit (Ycg1). Ycg1 expression is controlled at both the transcriptional and proteasomal level which results in high Ycg1 levels during mitosis and low Ycg1 levels during early interphase. Indeed, low Ycg1 levels during interphase correlate with low condensin formation, and increasing interphase Ycg1 expression resulted in higher condensin formation. Further, we found that low Ycg1 expression is necessary to avoid slow progression through interphase in budding yeast, where increased condensin may interfere with transcription and replication of DNA. Importantly, increasing the expression of the other condensin subunits had no impact on cell cycle progression. These observations are supported by recent findings in Drosophila, where overexpression of a single, rate-limiting condensin II subunit increased condensin-mediated interphase clustering of chromatin (Buster 2013). Together, these findings suggest that condensin function is limited during

interphase by the expression level of the rate limiting subunit. This mechanism, along with regulation of condensin activity and loading onto chromatin, precisely control condensin activity during the cell cycle.

CHAPTER II

Results

Preface

Data presented in the section are from the following publication:

Doughty TW, Arsenault HE, Benanti JA. Levels of Ycg1 Limit Condensin Function During the Cell Cycle. PLoS Genetics, 2016

Ycg1 protein and transcript levels are cell cycle-regulated.

Although the budding yeast condensin complex associates with chromatin throughout the cell cycle (Bhalla 2002, Lavoie 2002, Wang 2005, D'Ambrosio 2008), its activity increases substantially during mitosis. Previous reports have shown that this change in activity is due in part to increased phosphorylation (Sullivan 2004, St. Pierre 2009, Rebellet 2015), and to enhanced recruitment of the complex to a subset of sites in the genome (Wang 2005, D'Ambrosio 2008, Johzuka 2006, Peplowska 2014, Verzijlbergen 2014, Leonard 2015). Interestingly, several studies have also reported that transcription of the gene encoding the Cap-G subunit of condensin, *YCG1*, is cell cycle-regulated (Fig 2.1A) (Cho 1998, Pramila 2006, Landry 2014), with lower levels in G1 than mitosis. Additionally, Ycg1 protein levels have been reported to be lower in interphase than in mitosis (Lavoie 2004). This evidence suggests that regulation of Ycg1 levels may be an additional mechanism that coordinates condensin activity with the cell cycle. To

investigate this possibility further, we examined expression of Ycg1 mRNA and protein following release from a G1 arrest and found that they cycled similarly: expression increased as cells progressed through interphase, peaked during mitosis, and declined upon entry into the next G1 phase, similar to the mitotic cyclin Clb2 (Fig 2.1A, 2.1B). In contrast, none of the other subunits of the condensin complex displayed this dramatic fluctuation during the cell cycle, although Brn1 expression was modestly decreased in G1-arrested cells (Fig 2.1C, 2.1D). These observations, coupled with the fact that Ycg1 is essential for condensin function (Lavoie 2002, Lavoie 2004, D'Ambrosio 2008), suggest that regulation of Ycg1 levels during the cell cycle may be a previously uncharacterized mechanism that limits condensin function during interphase.

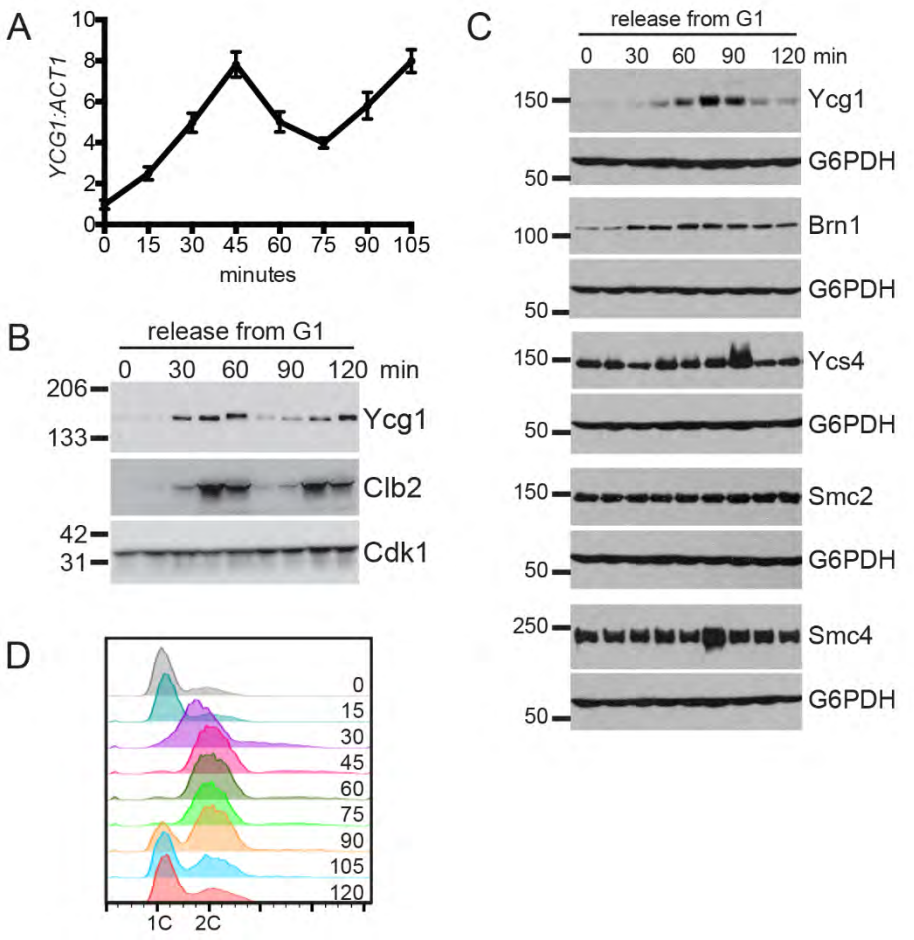


Figure 2.1. Ycg1 expression is cell cycle-regulated. (A) *YCG1* mRNA levels following release from G1 arrest. Wild-type cells (YBL320) were arrested in G1 with alpha-factor for 2 hours, released into the cell cycle, and gene expression changes were analyzed by RT-qPCR. *YCG1* levels are shown relative to *ACT1*. Time course samples were previously analyzed by DNA microarray in (Landry 2012). (**B)** Wild-type cells expressing Ycg1-GFP (YCG1-GFP) were arrested in G1 with alpha-factor for 3 hours then released into fresh medium, as in (A). Western blot samples were collected every 15 minutes. Clb2 is shown as a marker of mitosis and Cdk1 is shown as a loading control. **(C)** Wild-type strains expressing the individual condensin subunits tagged with a 3HA tag (YTD33, YTD82, YTD83, YTD84, YTD80) were arrested in G1 with alpha-factor for 3 hours, released into the cell cycle, and samples taken for Western blot and flow cytometry every 15 minutes. Alpha-factor was added back after 45 minutes to arrest cells in the subsequent G1 phase. **(D)** Representative plot showing DNA content following release from G1 arrest in (C), data is from the YTD33 time course. All other strains showed nearly identical plots. Note that the strains in used in parts A and B are in a different strain background than those used in parts C and D (S288C compared to W303), and the timing of cell cycle-progression differs slightly.

Ycg1 undergoes proteasomal degradation throughout the cell cycle.

The rapid decrease in Ycg1 levels after mitosis suggested that Ycg1 might also be regulated by proteolysis. To test this possibility and assay its stability, we monitored Ycg1 levels in asynchronous cells over time in the presence of the translation inhibitor cycloheximide, and found that Ycg1 was rapidly degraded (Fig 2.2A). Next, we asked whether other subunits of the complex were similarly regulated. To do this, each subunit of the complex was tagged with an identical 3HA tag, and their stabilities were compared in the same assay. This analysis revealed that Ycg1 is the least stable, and the least abundant, subunit of the condensin complex (Fig 2.2A).

Many cyclically expressed proteins are degraded by the ubiquitin proteasome system (UPS) (Benanti 2012), and Ycg1-ubiquitin conjugates were previously identified in a proteomic screen (Peng 2003), which suggested that Ycg1 may undergo ubiquitin-mediated degradation. Consistent with this possibility, proteasomal inhibition impaired Ycg1 turnover in asynchronous cells, confirming that the protein is regulated by the UPS (Fig 2.2B). Since Ycg1 is necessary for condensin function, and condensin function is essential for the completion of mitosis (Lavoie 2002, Lavoie 2004, D'Ambrosio 2008), we speculated that Ycg1 might be stable during mitosis. To test this, we arrested cells in G1 or mitosis, and monitored Ycg1 turnover (Fig 2.2C). We found that although there was more protein in mitosis, consistent with its increased transcription late in the cell cycle (Fig 2.1A), Ycg1 was degraded in both arrests. This observation suggests that

Ycg1 is degraded throughout the cell cycle, surprisingly, even during mitosis. Taken together, these data indicate that constitutive degradation, paired with cyclical transcription, leads to cell cycle-regulated expression of Ycg1.

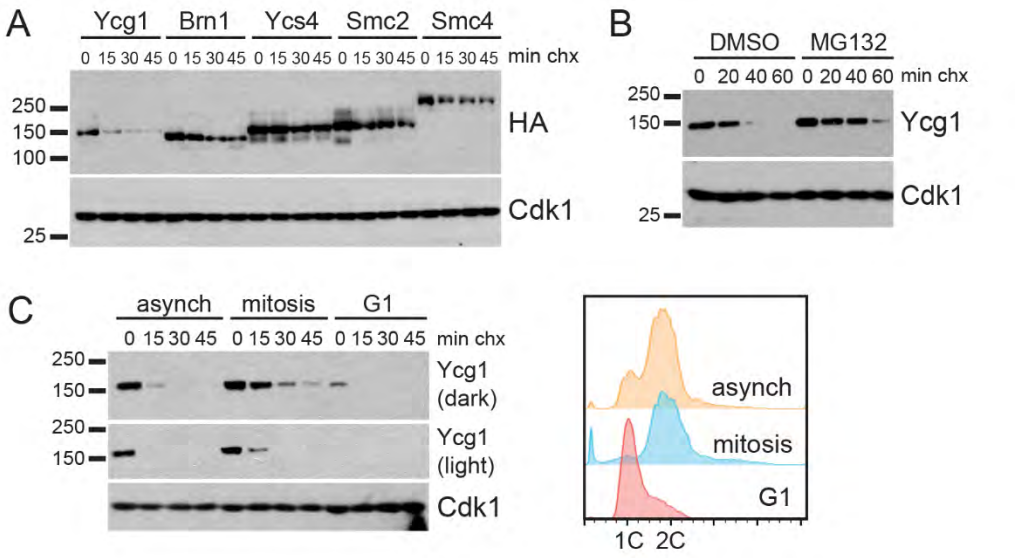


Figure 2.2. Ycg1 undergoes proteasomal degradation throughout the cell cycle. (A) Asynchronous cells harboring the indicated 3HA-tagged condensin subunits (YTD33, YTD82, YTD83, YTD84, YTD80) were treated with cycloheximide (chx) and samples were collected every 15 minutes. HA and Cdk1 Western blots are shown. **(B)** Asynchronous cells expressing Ycg1-3HA (YTD43) were incubated for 2 hours with DMSO (control) or the proteasome inhibitor MG132, then a cycloheximide-chase assay was performed, as in (A). **(C)** Cells expressing Ycg1-3HA (YTD33) were arrested with nocodazole (mitosis), alpha-factor (G1), or left untreated (asynch), and cycloheximide-chase assays were performed. Shown are Western blots examining Ycg1 (dark and light exposures of the same blot) and Cdk1 (left), as well as flow cytometry plots to confirm cell-cycle distributions (right).

The C-terminus of Ycg1 is necessary for its degradation.

Next, we sought to investigate the importance of cyclical Ycg1 expression for progression through the cell cycle. To do this, we engineered mutations in Ycg1 that blocked degradation. Most proteins that undergo ubiquitin-mediated degradation have short sequences termed degrons, which are essential for degradation. Many degron sequences are found in unstructured domains that are subject to other forms of regulation, such as phosphorylation (Holt 2012). Interestingly, the C-terminal domain of Ycg1 fits these criteria (St-Pierre 2009, Bazile 2010). Moreover, although this domain includes several phosphorylation sites that contribute to condensin activation during mitosis, this domain is not essential for viability (St-Pierre 2009), which allowed us to replace the endogenous copy of *YCG1* with alleles carrying mutations in this region. We first tested whether this domain was required for Ycg1 degradation and found that Ycg1 was completely stabilized when the C-terminal 63 amino acids were deleted (Fig 2.3A, Ycg1Δ973-1035). However, deletion of the C-terminal 50 amino acids had no effect on Ycg1 degradation (Ycg1Δ986-1035). These data suggested that Ycg1 turnover requires amino acids 973-985 and, consistent with this possibility, deletion of these amino acids was sufficient to stabilize the protein (Ycg1Δ973-985, Fig 2.3A). Additional deletions and truncations in the C-terminus were consistent with this conclusion (Fig 2.4A).

Since amino acids 973-985 lie within the conserved phosphoregulatory domain of Ycg1 (Fig 2.4A) (Bazile 2010), we endeavored to create a stable mutant

that minimally alters the sequence of this region. To do this we mutated features within this region that might contribute to degradation, including charged residues and putative phosphorylation sites (Fig 2.3B). We found that positively charged residues were necessary for Ycg1 degradation, with mutation of lysine-977 or arginine-978 having the greatest effect (Fig 2.3C). In contrast, mutation of negatively charged residues, or all serines and threonines in the region, had little to no effect on Ycg1 stability (Fig 2.4B). Although our data suggest that Ycg1 is degraded throughout the cell cycle (Fig 2.2C), we confirmed that the increased stability of Ycg1-K977A did not result from a change in cell-cycle distribution in the mutant strain by arresting cells in G1 or mitosis and assaying Ycg1 turnover. This analysis confirmed that Ycg1-K977A is more stable than wild-type Ycg1 in both phases of the cell cycle (Fig 2.3D).

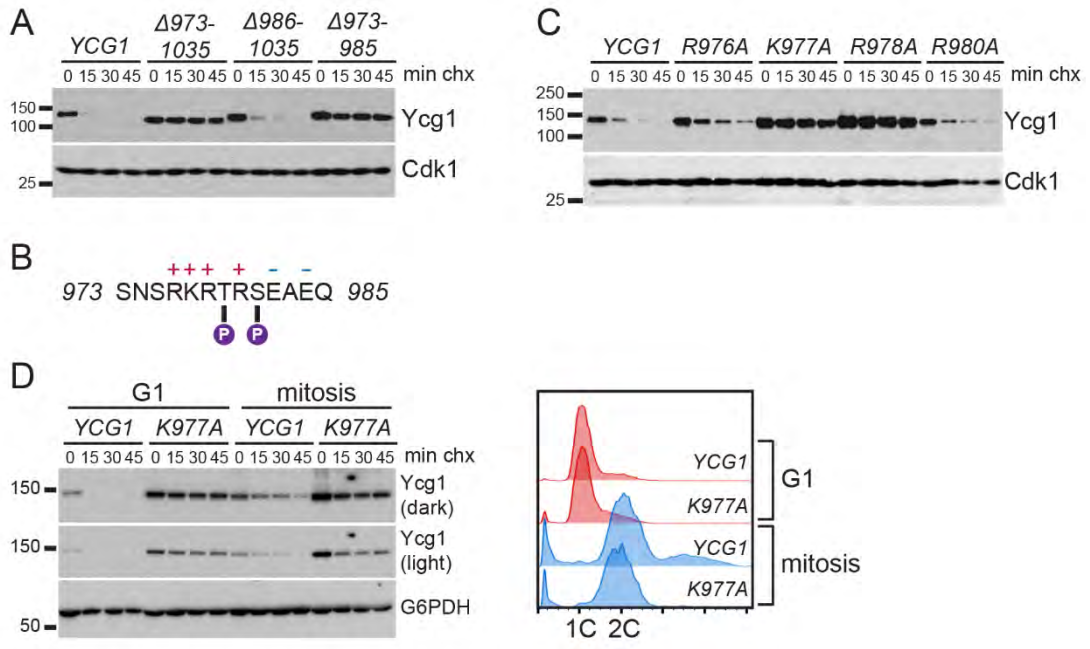


Figure 2.3. The C-terminus of Ycg1 regulates its degradation. (A) Cycloheximide-chase assay showing turnover of 3HA-tagged wild-type Ycg1 (YTD33) and the indicated deletion mutants (YTD36, YTD184, YTD128) in asynchronous cells. Also see Fig 2.4A for an illustration of the mutants. Western blots for HA and Cdk1 (loading control) are shown. **(B)** Sequence of Ycg1 amino acids 973-985 that regulate Ycg1 stability. Charged amino acids are indicated, as well as T979 and S981 which have been previously shown to be phosphorylated [14]. **(C)** Cycloheximide-chase assay of strains expressing the indicated 3HA-tagged Ycg1 proteins (YTD33, YTD200, YTD148, YTD201, YTD164) in asynchronous cells. Western blots for HA and Cdk1 (loading control) are shown. **(D)** *YCG1* (YTD33) and *ycg1-K977A* (YTD148) strains were arrested in G1 with alpha-factor or in mitosis with nocodazole for 3 hours and cycloheximide-chase assays performed. Western blots for HA and G6PDH (loading control) are shown. Flow cytometry plots (right) confirm cell-cycle arrests.

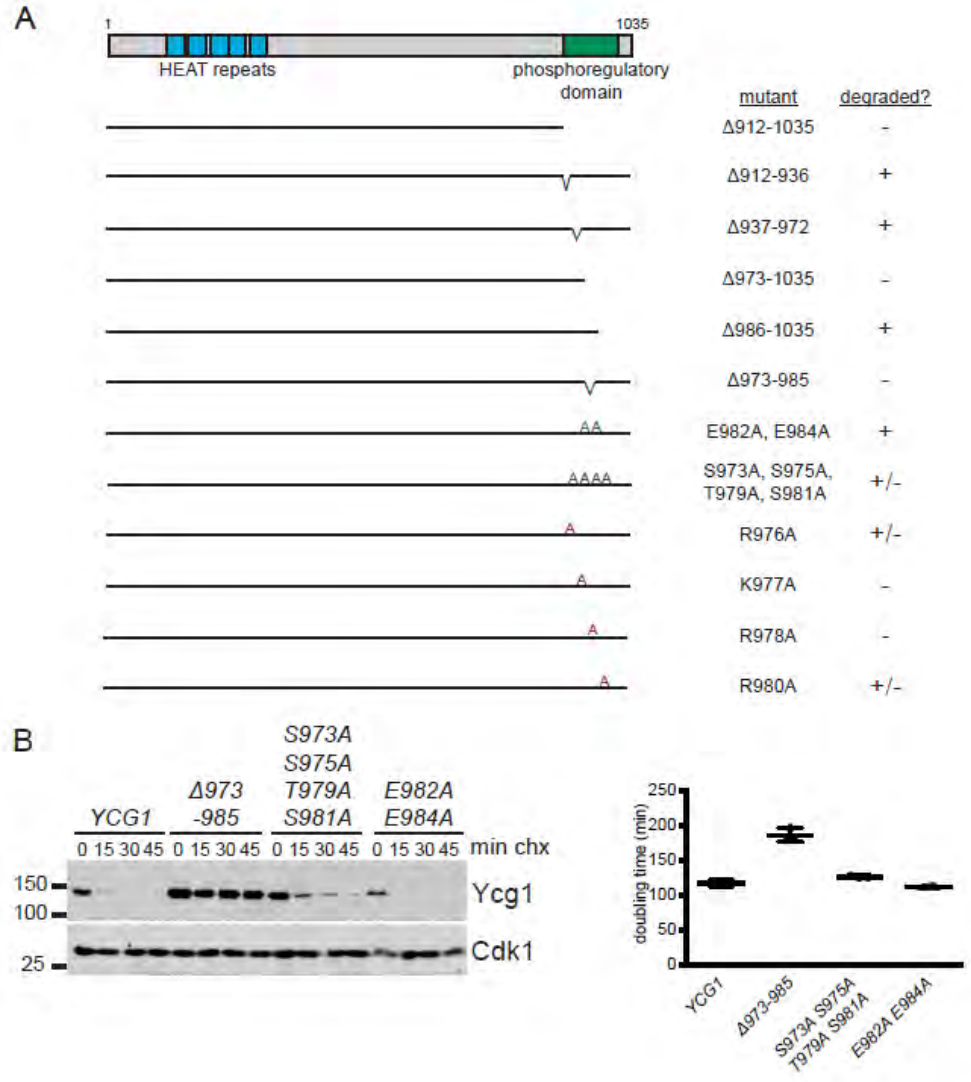


Figure 2.4. Summary of Ycg1 C-terminal mutations and their effects on Ycg1 stability. **(A)** Diagram of all Ycg1 mutations tested. Stability of each mutant was assayed by cycloheximide-chase assay: (+) protein is degraded similar to wild-type, (+/-) modest stabilization compared to wild-type Ycg1, and (-) protein does not get degraded. **(B)** Cycloheximide-chase assay (left) and doubling time analysis (right) of strains expressing wild-type Ycg1 (YTD33), or proteins that harbor mutations in putative phosphorylation sites or acidic amino acids (YTD128, YTD199, YTD176). Mutation of threonine and serine residues results in a modest increase in stability, whereas mutation of acidic residues has no effect on protein turnover. Neither mutant increases the doubling time of cells.

Constitutive Ycg1 expression delays cell-cycle entry.

The prevailing model suggests that chromosome condensation needs to be reversed after mitosis so that chromosomes become accessible for essential DNA-dependent processes during interphase, such as replication and transcription. Since Ycg1 is downregulated after mitosis, we posited that interference with this regulation might impact cell-cycle progression. To test this, we analyzed the proliferation rate of each of the strains expressing point mutations that stabilize Ycg1. Interestingly, we observed a modest increase in doubling time in mutants that partially blocked Ycg1 turnover, and a much larger increase in doubling time in mutants that fully blocked turnover (Figs 2.5A, 2.4B). These data show a correlation between increased Ycg1 expression and decreased proliferation rate, suggesting that Ycg1 downregulation after mitosis may be important for cell-cycle progression.

Next, we asked whether the decreased proliferation rate that we observed in cells expressing stable Ycg1 resulted from a delay at a specific point in the cell cycle. Strains expressing Ycg1 or Ycg1-K977A were synchronized in G1 phase and released. Cell-cycle progression was then followed by flow cytometry and Ycg1 levels were monitored by Western blot. In contrast to the wild-type protein, Ycg1-K977A was expressed at a constant level throughout the cell cycle (Fig 2.5B, top), demonstrating that degradation is necessary for cell cycle-dependent changes in Ycg1 levels. Notably, *ycg1-K977A* strains exhibited delayed progression from G1 into S phase (Fig 2.5B, bottom), consistent with the possibility

that failing to downregulate condensin might interfere with progression through interphase.

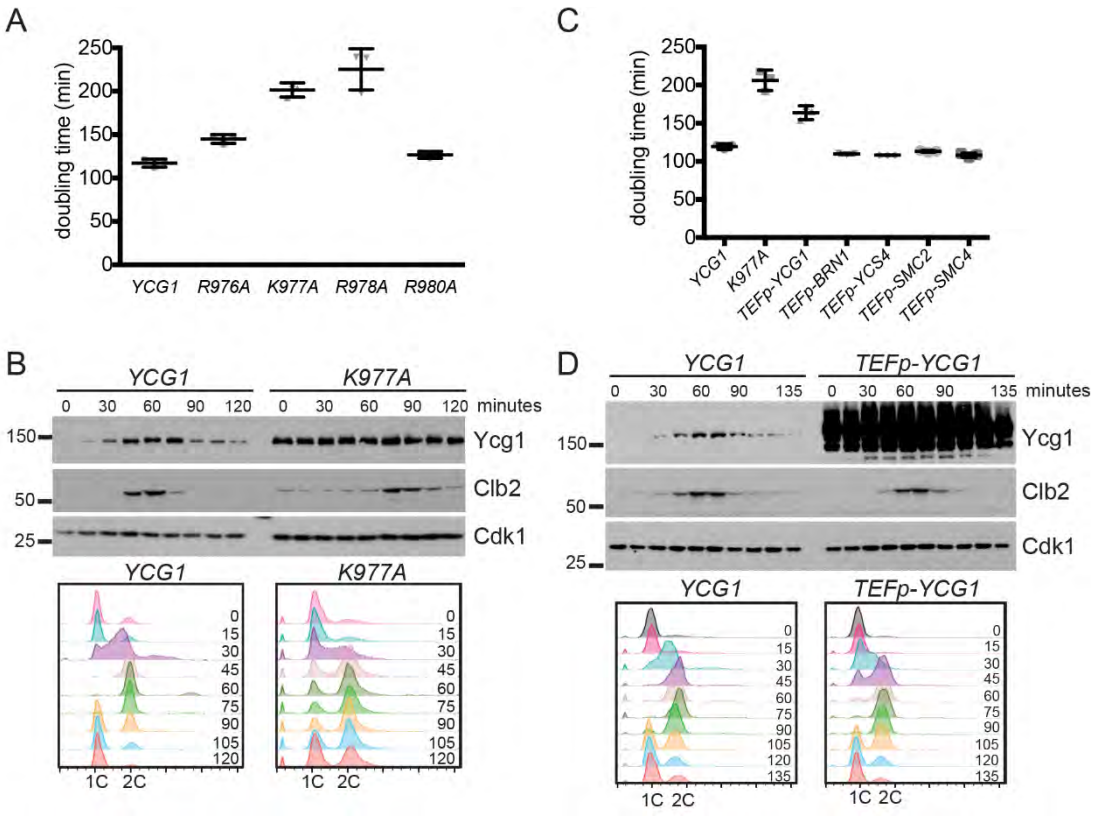


Fig 2.5. Constitutive expression of Ycg1 delays progression through the cell cycle. (A) Doubling time of strains expressing the indicated Ycg1 proteins from the endogenous locus (YTD33, YTD200, YTD148, YTD201, YTD164). Mean doubling time from 3 independent experiments, +/- 1 standard deviation, are shown. **(B)** Strains expressing 3HA-tagged Ycg1 (YTD33) or Ycg1-K977A (YTD148) from the endogenous locus were arrested in G1 with alpha-factor for 3 hours and then released into the cell cycle. Alpha-factor was added back after 45 minutes to prevent cells from entering a second cycle. Western blots of 3HA-tagged Ycg1, Clb2 and Cdk1 are shown (top). Flow cytometry plots (bottom) illustrate the delayed progression of *ycg1-K977A* cells into S phase (compare 30 and 45-minute time points). **(C)** Doubling time of wild-type (YTD33) and *ycg1-K977A* strains (YTD148) were compared to strains overexpressing each of the indicated condensin subunits from the *TEF1* promoter (YTD336, YTD337, YTD353, YTD349, YTD362). Mean doubling times from 3 independent experiments are shown, +/- 1 standard deviation. **(D)** *YCG1* (YTD276) and *TEFp-YCG1* (YTD361) strains were arrested in metaphase with a *MET3p-CDC20* shut-off allele for 3 hours, then released into alpha-factor for 2 hours to synchronize cells in G1. Alpha-factor was added back 45 minutes after release from G1 arrest to prevent cells from entering a second cycle. Western blots of 3HA-tagged Ycg1, Clb2 and Cdk1 are shown (top). Flow cytometry plots (bottom) demonstrate the delayed progression of *ycg1-K977A* cells into S phase (compare 30 and 45-minute time points).

Haploid *ycg1-K977A* strains are viable, confirming that the allele encodes a functional protein. However, the K977A mutation falls in a domain of Ycg1 that is required for maximal condensin activity (St-Pierre 2009), raising the possibility that this mutation might both increase Ycg1 expression and reduce its function. To address this possibility, we performed additional characterization of *ycg1-K977A* strains. First, we confirmed that the interaction between Ycg1-K977A and the other subunits of condensin was not impaired (Fig 2.6A). In addition, we used an established rDNA reporter assay (Smith 1999) to investigate whether *ycg1-K997A* cells exhibited defects in rDNA silencing, or increased recombination at the rDNA locus, both of which are phenotypes exhibited by condensin loss-of-function mutants (Freeman 2000, Bhalla 2002). We found that *ycg1-K977A* cells were similar to wild-type cells in this assay (Fig 2.6B). Moreover, the proliferation defect in *ycg1-K977A* strains could not be rescued by the addition of a second copy of *YCG1* integrated at the *URA3* locus, suggesting that the growth defect is not the result of reduced function of the mutant (Fig 2.6F). Although these assays suggested Ycg1-K977A is functional, we observed that multiple isolates of haploid *ycg1-K977A* strains exhibited non-uniform colony size (Fig 2.6C), exhibited increased sensitivity to the replication inhibitor hydroxyurea (HU) (Fig 2.6D) (a phenotype that has been reported for strains expressing hypomorphic alleles of condensin subunits in fission yeast (Aono 2002), and showed increased sensitivity to the microtubule poison benomyl (Fig 2.6E). Moreover, we had difficulty generating haploid strains that expressed Ycg1-K977A and had an epitope tag on

any other subunit of the condensin complex. Together, these findings suggested that the K977A mutation might reduce Ycg1 function, in addition to stabilizing the protein.

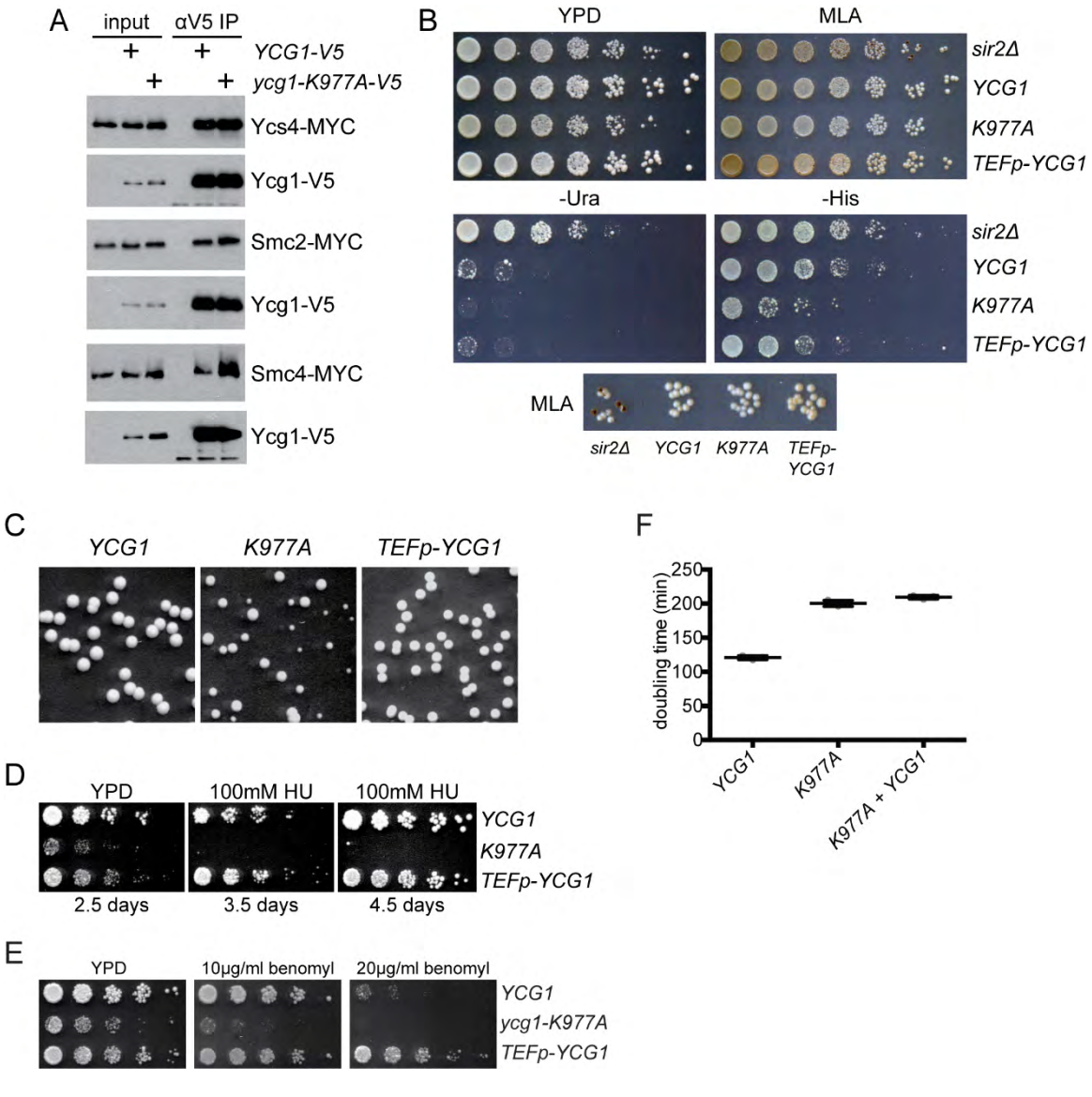


Figure 2.6. Analysis of *ycg1-K977A* strains. (A) Heterozygous diploid cells expressing one allele of 3V5-tagged Ycg1 or Ycg1-K977A, as well as one allele of 13Myc-tagged Ycs4 (YTD284, YTD268, YTD269), Smc2 (YTD274, YTD285, YTD267), or Smc4 (YTD275, YTD286, YTD255) were used to assay condensin complex formation. Ycg1 was immunoprecipitated via its 3V5 tag in each strain, and the association of each other tagged subunit assayed by Western blot against the Myc tag. Ycg1-K977A associates with each other subunit as well as wild-type Ycg1. (Continued on next page.)

Figure 2.6. Analysis of *ycg1-K977A* strains. (continued) (B) rDNA silencing and stability were assayed using previously described strains that harbor multiple markers integrated into the rDNA locus [44]. Wild-type (YHA212), *ycg1-K977A* (YHA214), and *TEFp-YCG1* (YHA215) strains were compared to a *sir2*Δ strain (JS576) previously shown to have a silencing defect and to exhibit increased recombination at the rDNA locus [44]. In this assay, a silencing defect is detected by growth on –Ura plates and increased rDNA recombination is evident by dark brown and/or sectored colonies on MLA plates. Growth on –His plates confirms the presence of the *URA3*/*HIS3* cassette. Stabilization or overexpression of *YCG1* does not result in either phenotype, confirming there is no defect in rDNA regulation in these strains. **(C)** Wild type (YTD33), *ycg1-K977A* (YTD148) and *TEFp-YCG1* (YTD336) strains were grown on YPD plates. Images show representative colony sizes. **(D)** Strains from (C) were diluted five-fold and spotted onto a YPD plate, or YPD plates containing 100mM hydroxyurea (HU), and incubated at 30ºC for the indicated number of days. **(E)** Strains from (C) were diluted 5-fold and spotted onto a YPD plate, or YPD plates containing the indicated concentration of benomyl. Notably, *TEFp-YCG1* cells exhibit resistance to high concentrations of benomyl, which could result from an increase in condensin association with centromeres in this strain (Fig 8A). **(F)** An extra copy of *YCG1* expressed from its own promoter was integrated into the *URA3* locus in the *ycg1-K977A* strain. The doubling time of the resulting strain (YTD430) was compared to the parental strains (YTD148) and a wild-type strain (YTD33). Shown is the average doubling time from three independent experiments +/- 1 standard deviation.

To distinguish between these effects and determine whether the increased expression of Ycg1-K977A was the primary cause of the proliferation defects described above, we disrupted cell cycle-regulation of Ycg1 levels in an alternative way, using the constitutive *TEF1* promoter to drive Ycg1 expression, and assayed for changes in proliferation rate. Asynchronous *TEFp-YCG1* strains expressed approximately 4-fold more Ycg1 protein than wild-type cells (Fig 2.12A), and also displayed an increase in doubling time (Fig 2.5C). Furthermore, both *ycg1-K977A* and *TEFp-YCG1* strains showed a delay in G1/S progression (Fig 2.5B, 2.5D), and both exhibited sensitivity to temperature stress (Fig 2.7A). Together, these data argue that increasing Ycg1 abundance is sufficient to delay the cell cycle and decrease proliferation rate. Notably, overexpression of Ycg1 did not result in heterogeneous colony size or sensitivity to HU or benomyl (Fig 2.6), which suggests that these phenotypes of the *ycg1-K977A* strain may result from its reduced function, and not increased expression of the stable protein.

The delay in cell-cycle progression described above could be the result of a delay in the G1/S transition and/or an inhibition of DNA replication in mutant strains. To determine whether the transition from G1 into S phase was delayed, we monitored budding, since bud formation is triggered by the wave of transcription that occurs at the G1/S transition, but is independent of replication initiation (Haase 1999). Interestingly, the delay in DNA synthesis in *ycg1-K977A* and *TEFp-YCG1* strains correlated with a proportional delay in budding (Fig 2.7B, 2.7C), indicating that these strains exhibit a delay in entering S phase. The delay was most evident

22.5 minutes after release from G1, when wild-type cells were in S phase and *ycg1-K977A* and *TEFp-YCG1* strains were largely still in G1 (Fig 2.7C). Consistent with a previous report (D'Ambrosio 2008), this delay was not observed in the condensin temperature-sensitive mutants *ycg1-2* and *brn1-9* (Li 2011) when they were released from G1 arrest at the restrictive temperature (Fig 2.8), confirming that the G1/S delay observed upon Ycg1 overexpression is distinct from condensin loss of function.

Chromosomes decondense in telophase, so condensin activity must decrease at the end of mitosis. One possibility is that the increased Ycg1 levels in *ycg1-K977A* and *TEFp-YCG1* strains might impair chromatin decondensation, which could induce an additional cell-cycle delay when cells exit from mitosis. We tested for this possibility by synchronizing cells in metaphase with a *CDC20* shut-off allele and monitoring progression of each strain into G1 phase by flow cytometry. Both strains exited mitosis with kinetics similar to a wild-type strain (Fig 2.7D), arguing against this possibility. We also assayed chromosome condensation directly, by examining the structure of the rDNA locus, which undergoes dramatic compaction during mitosis that can be visualized in chromosome spreads (Guacci 1997, Lavoie 2002, Lavoie 2004). Cells were arrested in both metaphase and G1, the rDNA was visualized by Net1 staining on chromosome spreads, and condensation scored as previously described (Lavoie 2004, D'Ambrosio 2008). Notably, there was no significant difference in rDNA conformation between wild-type and *TEFp-YCG1* strains, in either metaphase or

G1-arrested cells (Fig 2.9). Together, these results argue that increasing Ycg1 expression does not alter rDNA condensation, or delay exit from mitosis.

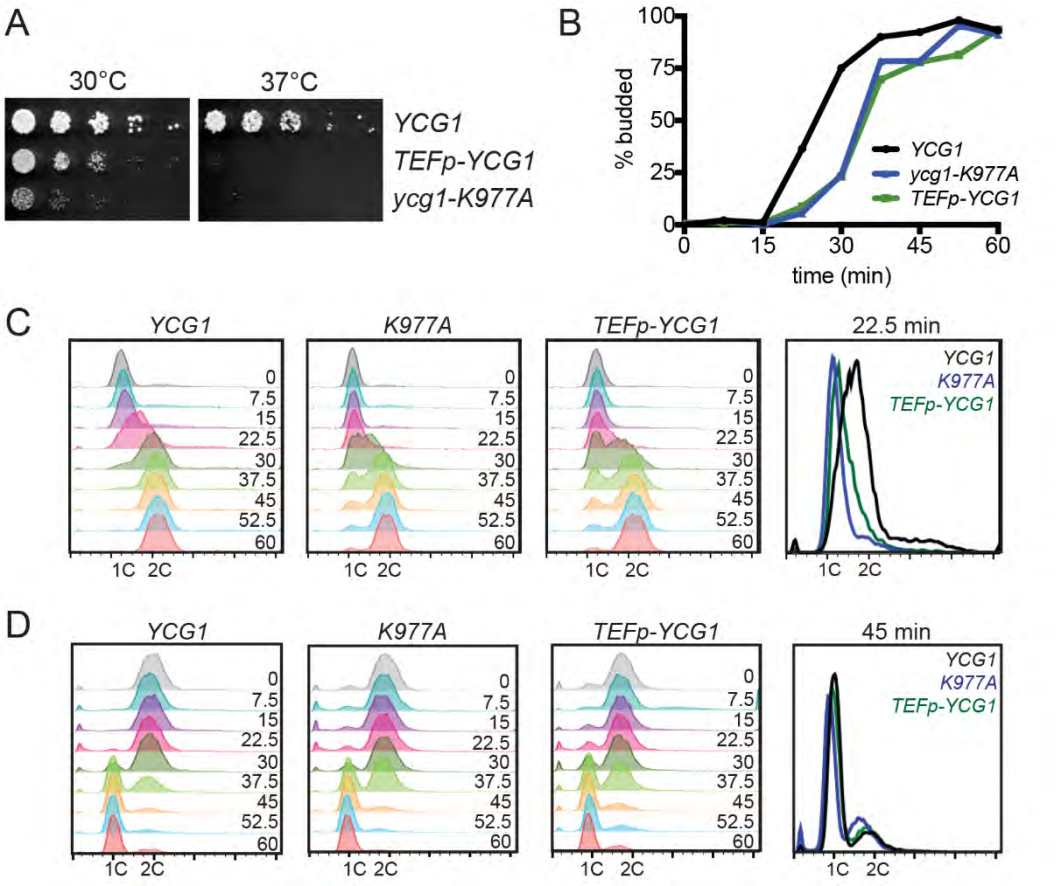


Fig 2.7. Increased expression of Ycg1 delays entry into S-phase. (A) 5-fold dilutions of strains with the indicated genotypes (YTD33, YTD148, YTD336) were plated on YPD and incubated at the indicated temperatures until the colonies in the wild-type strain were of similar size. **(B-C)** Wild-type (YTD276), *ycg1-K977A* (YTD290), and *TEFp-YCG1* (YTD361) strains were synchronized in G1 as in (Fig 4D) and samples fixed at 7.5 minute intervals after release to measure the percentage of budded cells (B), and progression through S phase by flow cytometry (C). **(D)** Wild-type (YTD276), *ycg1-K977A* (YTD290), and *TEFp-YCG1* (YTD361) strains were synchronized in metaphase with a *MET3p-CDC20* shut-off allele then released into the cell cycle and samples were fixed at 7.5 minute intervals after release. Progression into G1 phase was measured by flow cytometry.

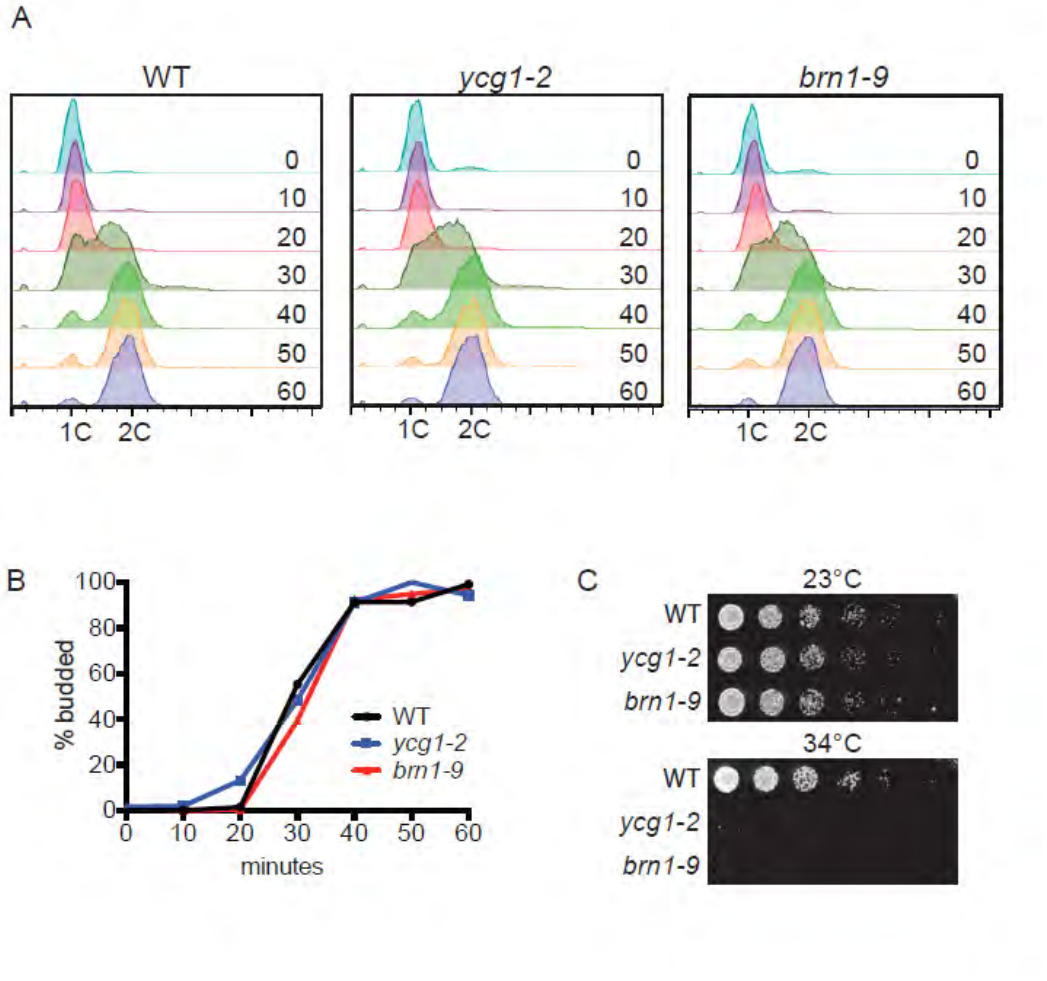


Figure 2.8. Inactivation of condensin does not delay the G1/S transition. (A-B) Wild type (MW836a), *ycg1-2* (Y10100), and *brn1-9* (Y9804) strains were arrested in G1 with alpha-factor for 4 hours at 23°C (with and additional aliquot of alpha-factor added after 2 hours) and released into fresh medium without alpha-factor at 34°C. Samples were fixed every 10 minutes for 60 minutes following release. DNA replication was monitored by flow cytometry (A), and number of budded cells counted (B), at each time point. **(C)** 5-fold dilutions of the strains from (A) were plated on YPD plates and incubated at the indicated temperatures. Both *ycg1-2* and *brn1-9* strains arrest at 34°C.

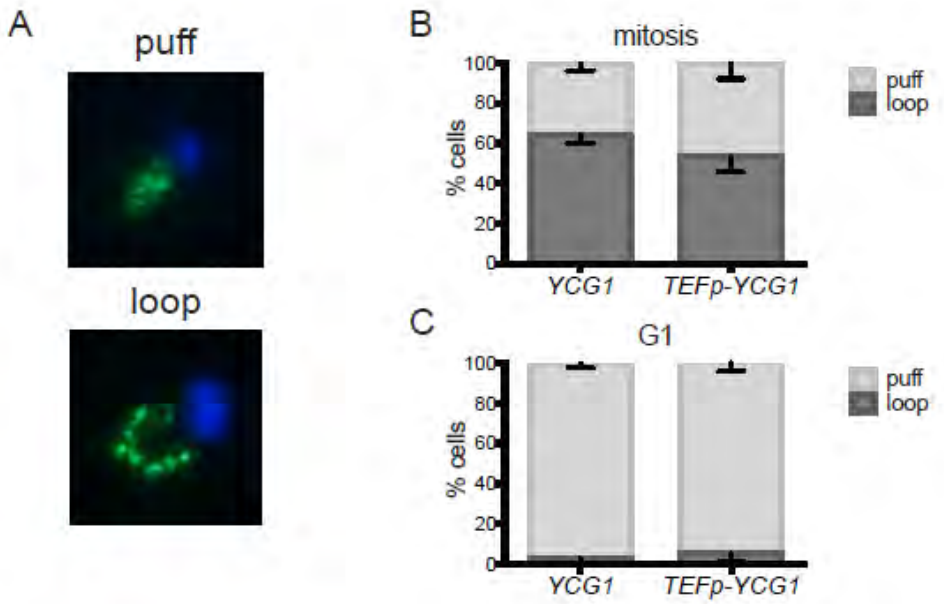


Figure 2.9. Analysis of rDNA condensation upon *YCG1* overexpression. (A) Representative images of rDNA morphology as visualized by chromosome spreads. Cells were arrested in G1 by the addition of alpha-factor, or in metaphase by the addition of 20µg/ml nocodazole, for 3 hours. Spheroplasts were prepared and chromosomes spread on glass slides. Chromosomes were stained with DAPI and the rDNA was visualized by immunofluorescence to detect 3V5-tagged Net1, which is enriched on nucleolar DNA [22,47]. A puff represents decondensed DNA, whereas a loop represents condensed rDNA. **(B)** Percentages of rDNA puffs and loops in wild-type (YJB653) and *TEFp-YCG1* (YJB651) cells arrested in metaphase. In each experiment at least 130 cells were scored. Shown are the mean percentages +/- 1 standard deviation from n=4 (YJB653) and n=3 (YJB651) experiments. An unpaired t-test was used to confirm that there is no statistically significant difference between strains. **(C)** Percentages of rDNA puffs and loops in wild-type (YJB653) and *TEFp-YCG1* (YJB651) cells arrested in G1. In each experiment at least 100 cells were scored. Shown are the mean percentages +/- 1 standard deviation from n=4 (YJB653) and n=3 (YJB651) experiments. An unpaired t-test was used to confirm that there is no statistically significant difference between strains.

Ycg1 levels are limiting for condensin recruitment to chromatin.

Our comparison of the expression levels of condensin subunits indicates that Ycg1 is expressed at lower levels than the other subunits (Figs 2.2A, 2.10). In addition, Ycg1 is the only condensin subunit that cycles (Fig 2.1C). These findings raise the possibility that Ycg1 levels might be limiting for complex formation. If this is the case, then overexpression of other subunits of the complex should not impair cell-cycle progression in the way that overexpression of Ycg1 does. To test this hypothesis, we integrated the *TEF1* promoter upstream of the other four subunits of the condensin complex. Importantly, although each condensin subunit was overexpressed in these strains to similar levels (Fig 2.10A), increasing expression of no other condensin subunit led to an increase in doubling time (Fig 2.5C). Moreover, while asynchronous *TEFp-YCG1* cells displayed an increased fraction of cells in G1 phase, consistent with a G1/S delay, there was no change in the fraction of G1 cells upon overexpression of any other condensin subunit (Fig 2.10B). These data are in agreement with the model that Ycg1 is the limiting subunit for condensin function.

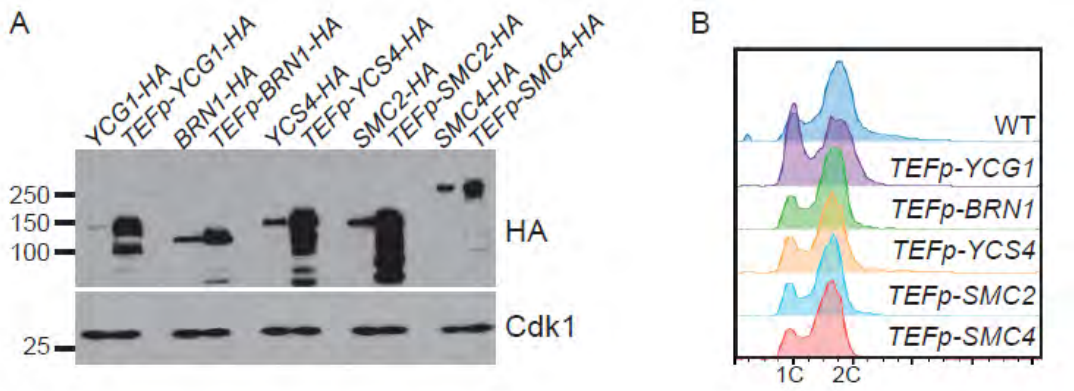


Figure 2.10. Expression of condensin subunits in *TEF1* promoter knock-in strains. (A) Western blot showing relative expression of each 3HA-tagged condensin subunit in asynchronous wild-type (YTD33, YTD82, YTD83, YTD84, YTD80) and *TEF1p* knock-in (YTD336, YTD337, YTD353, YTD349, YTD362) strains. Cdk1 is shown as a loading control. **(B)** DNA content of asynchronous cultures as measured by flow cytometry showing the cell-cycle distributions of asynchronous cultures of the strains from (A). Note that overexpression of Ycg1, but not any other condensin subunit, results in a larger fraction of cells in G1 phase, consistent with a G1/S delay.

Ycg1 has not been shown to function on its own, or as part of any protein complex other than condensin. Therefore, we hypothesized that increased Ycg1 expression slowed G1/S progression as a result of increased condensin complex during G1 phase. Notably, this hypothesis makes two predictions: first, that the amount of intact condensin complex varies based on cell-cycle position, and second, that modulation of Ycg1 levels is necessary to establish this variation. To test these possibilities, we assayed for changes in condensin subunit interactions in different cell-cycle phases. First, we arrested cells in G1 phase or mitosis, immunoprecipitated different subunits of the condensin complex, and determined whether more Ycg1 associated with each subunit in mitosis than in G1 phase. Importantly, more Ycg1 co-immunoprecipitated with other condensin subunits in mitosis than G1 (Fig 2.11, compare lanes 5 and 11 in each panel), confirming the level of intact condensin complex varies in different cell-cycle phases.

We simultaneously performed co-immunoprecipitation experiments in *TEFp-YCG1* strains to determine if preventing the downregulation of Ycg1 led to an increase in the amount of intact condensin complex. Notably, more Ycg1 was associated with other subunits of the complex in the *TEFp-YCG1* background compared to wild-type cells in G1 phase (Fig 2.11, compare lanes 5 and 6 in each panel). In mitotic cells we observed a small increase in Ycg1 interaction over the already high levels in wild-type cells when Ycg1 was overexpressed (Fig 2.11, compare lanes 11 and 12 in each panel). These data show that overexpression of

Ycg1 increases condensin subunit interactions considerably in G1, when Ycg1 is limiting, and less so during mitosis, when Ycg1 levels peak.

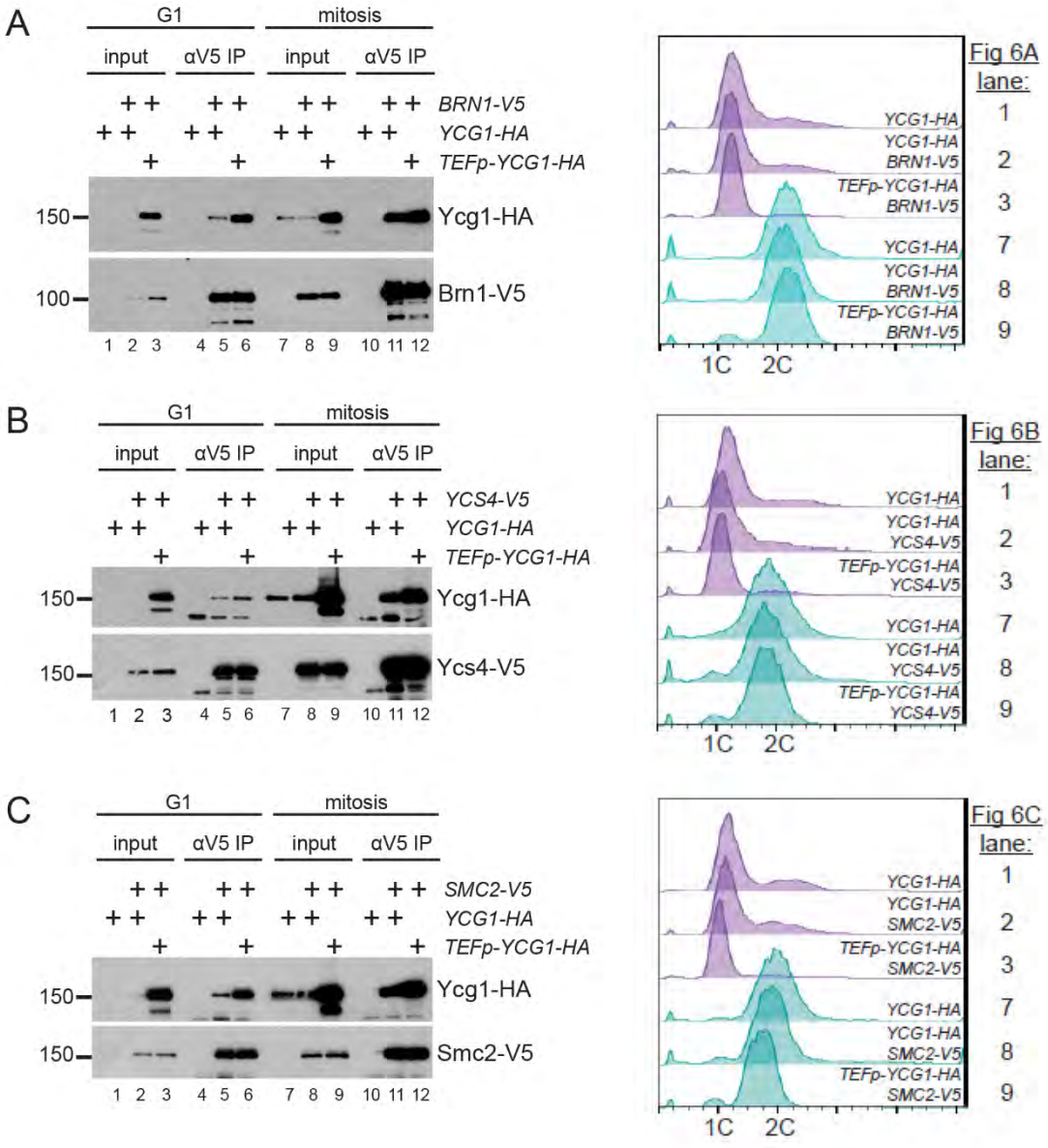
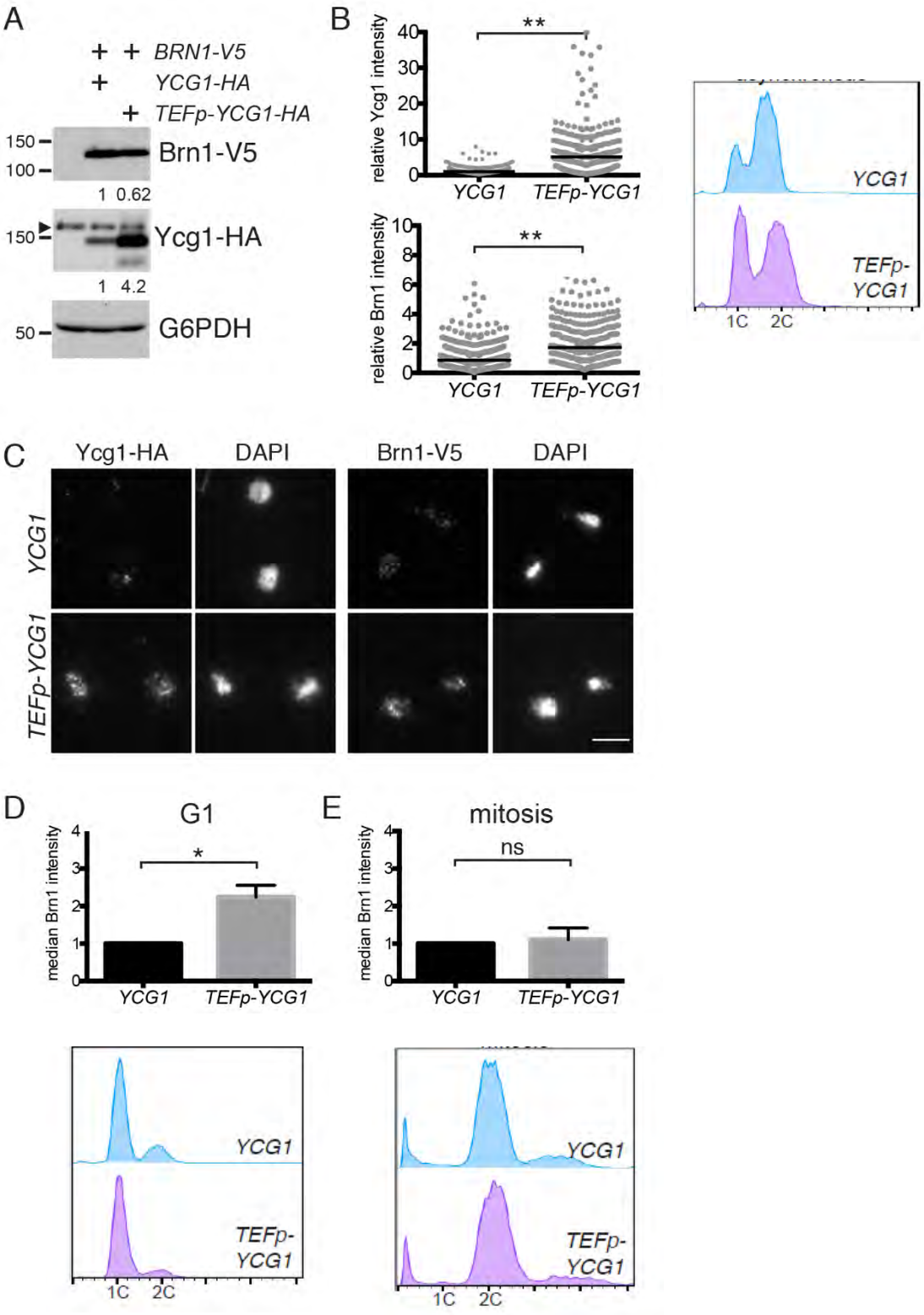


Fig 2.11. Cell cycle-regulation of Ycg1 limits condensin complex formation in G1 phase. *YCG1* (YTD302, YTD394, YTD396) and *TEFp-YCG1* (YTD355, YTD395, YTD397) strains were synchronized in mitosis with a *MET3p-CDC20* shut-off allele, or in G1 by releasing from the mitotic arrest into medium containing alpha-factor for 2 hours. Condensin complexes were then immunoprecipitated from arrested cells with antibodies against a 3V5 tag on Brn1 **(A)**, Ycs4 **(B)**, or Smc2 **(C)**, and Ycg1 association with each subunit was assayed by Western blot for the 3HA tag on Ycg1. In all experiments, a strain lacking the V5 tag on Brn1 (YTD276) was used as a negative control. Flow cytometry data verifying cell-cycle arrest is shown to the right of each respective experiment.

A previous study demonstrated that Ycg1 is required to recruit other condensin subunits to chromatin (Piazza 2014). Therefore, we investigated whether the chromatin association of the Brn1 subunit was increased in *TEFp-YCG1* cells by quantifying the amount of Brn1 that associated with chromosomes in a chromosome spread assay (Lavoie 2002, D'Ambrosio 2008, Cuylen 2011). Notably, although overexpression of Ycg1 did not increase total cellular levels of Brn1 (Fig 2.12A), the association of Brn1 with chromatin increased in *TEFp-YCG1* cells (Fig 2.12B-C). This increase in Brn1 association was observed in both asynchronous cells and cells arrested in G1 phase (Fig 2.12D). In contrast, there was no significant increase in bulk chromatin association of Brn1 in mitotic cells (Fig 2.12E). These results are consistent with the observation that increasing Ycg1 expression leads to a greater increase in the levels of intact complex in G1 than in mitosis (Fig 2.11), and support the possibility that an increase in the association of condensin with chromosomes in G1 phase delays cell-cycle entry.


A
+ + BRN1-V5
+ YCG1-HA
+ TEFp-YCG1-HA
150
100
Brn1-V5
1 0.62
150
Ycg1-HA
1 4.2
50
G6PDH
B
relative Ycg1 intensity
**
YCG1
TEFp-YCG1
relative Brn1 intensity
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YCG1
TEFp-YCG1
YCG1
TEFp-YCG1
1C 2C
C
Ycg1-HA
DAPI
Brn1-V5
DAPI
YCG1
TEFp-YCG1
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G1
median Brn1 intensity
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YCG1
TEFp-YCG1
YCG1
TEFp-YCG1
1C 2C
E
mitosis
median Brn1 intensity
ns
YCG1
TEFp-YCG1
YCG1
TEFp-YCG1
1C 2C

Fig 2.12. Ycg1 is limiting for condensin recruitment to chromatin. (A) Western blot showing total levels of Ycg1-3HA and Brn1-3V5 proteins in asynchronous wild-type (YTD297) and *TEFp-YCG1* (YTD342) cells. G6PDH is shown as a loading control. Relative expression of Ycg1 and Brn1, normalized to G6PDH, are indicated beneath each blot. **(B)** Representative experiment showing quantification of Ycg1-3HA and Brn1-3V5 staining on chromosome spreads from cells in (A). At least 190 cells in each sample were quantified, with the median intensity indicated by the black line. Values are shown relative to the median intensity in wild-type cells. Both proteins are significantly enriched on chromatin in *TEFp-YCG1* cells, as determined by an unpaired t-test (**p<0.0001) (left). FACS corresponding to this experiment are shown (right) **(C)** Representative images of Ycg1-3HA and Brn1-3V5 staining on chromosome spreads from the experiment shown in (B). Scale bar represents 5 μm. **(D)** Wild-type (YTD297) and *TEFp-YCG1* (YTD342) cells were arrested in G1 with alpha-factor (bottom) and chromosome spreads performed as in (B) (top). Shown is the average of the median intensity values (normalized to the median in wild-type) from 3 experiments, +/- 1 standard deviation. Brn1 is significantly enriched on chromatin in *TEFp-YCG1* cells, as determined by an unpaired t-test (*p<0.05). **(E)** Brn1-3V5 staining on chromosome spreads as in (D) except strains were arrested in nocodazole (top). An unpaired t-test was used to confirm that there is no significant difference between strains (ns). Flow cytometry plots confirming cell-cycle positions for all experiments are shown (bottom).

Although condensin associates with chromosomes throughout the cell cycle, its enrichment at many of its best characterized binding sites (including the rDNA, centromeres, and telomeres) is substantially higher in mitosis than in G1 (Wang 2005, Wang 2006, Johzuka 2006, Verzijlbergen 2014, Leonard 2015). Notably, each of these classes of binding sites requires mitosis-specific factors to stimulate this increase in condensin recruitment (Clemente-Blanco 2011, Verzijlbergen 2014, Robellet 2015), which raises the question of whether or not Ycg1 overexpression leads to increased condensin binding to these specific loci during interphase. To address this question, we used chromatin immunoprecipitation and quantitative PCR (ChIP-qPCR) to quantify Brn1 recruitment to a representative set of these sites (Haeusler 2008, D'Ambrosio 2008, Clemente-Blanco 2011, Verzijlbergen 2014). Interestingly, in asynchronous *TEFp-YCG1* cells, Brn1 binding increased at centromeric and telomeric loci, but not the rDNA (Fig 2.13A).

We next used ChIP-qPCR to examine Brn1 recruitment to mitotic binding sites in cells that were arrested in G1 and metaphase, in order to directly compare binding at these sites to bulk chromatin binding that we had measured using chromosome spreads (Fig 2.12). These experiments led to two interesting observations. First, consistent with the results of the chromosome spread experiments, Brn1 binding to mitotic sites was not significantly elevated in metaphase cells upon overexpression of Ycg1 (Fig 2.13B). (Although binding at centromeres tended to be slightly elevated in *TEFp-YCG1* cells, the data did not

reach statistical significance, and a modest reduction in binding to the rDNA was observed.) The second conclusion from these data is that although Brn1 bound to the rDNA, centromeres, and a telomere in metaphase, binding at each of these sites was reduced to background levels in both wild-type and *TEFp-YCG1* strains that were arrested in G1 (Fig 2.13B). This result indicates that although total Brn1 binding to chromosomes is elevated in *TEFp-YCG1* strains in G1 (Fig 2.12D), the complex is not enriched at mitosis-specific target sites. In addition, the increased binding of condensin to centromeres and telomeres that is seen in asynchronous *TEFp-YCG1* cells is likely to result from increased binding at a point in the cell cycle other than G1 or metaphase.

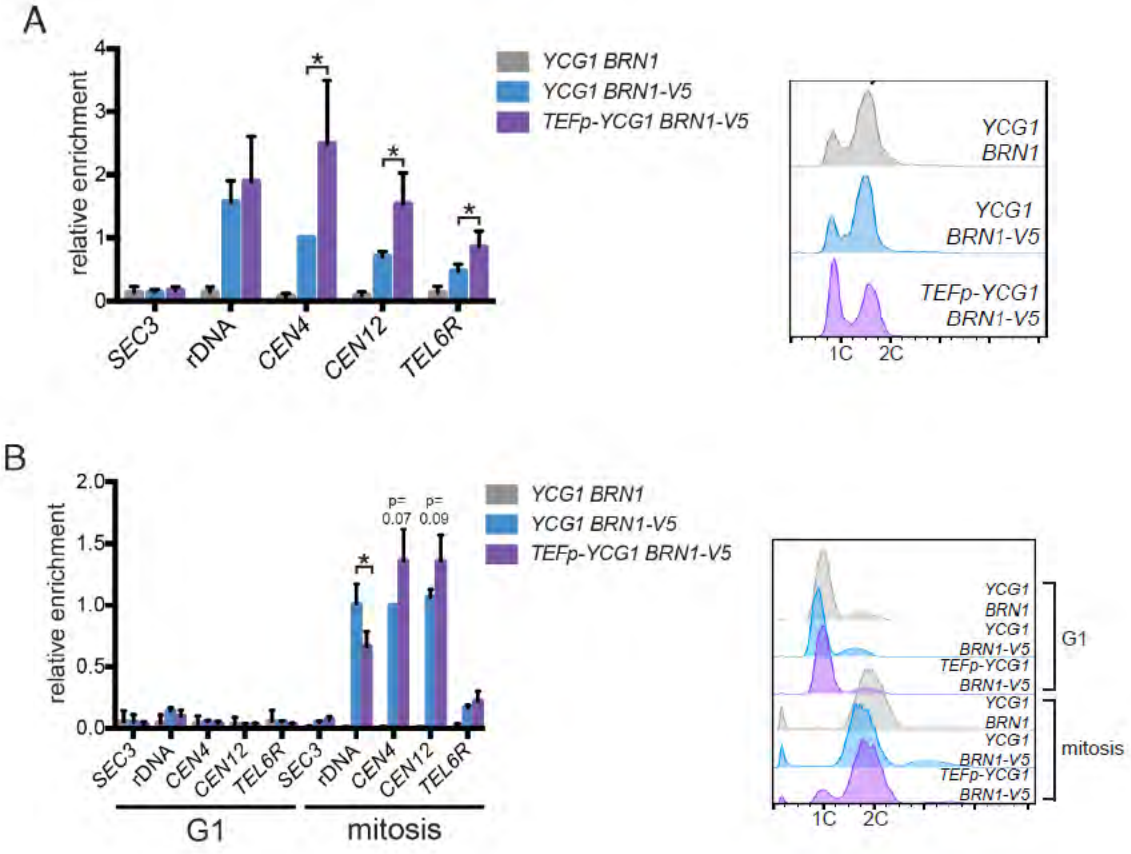


Figure 2.13. Increasing Ycg1 expression in G1 does not promote condensin binding to mitosis-enriched sites. **(A)** ChIP-qPCR of Brn1-3V5 from asynchronous *YCG1* (YTD297) and *TEFp-YCG1* (YTD342) strains, as well a wild-type strain lacking the 3V5 tag (YTD33) as a negative control. Brn1 enrichment at the rDNA (replication fork barrier), the centromeres of chromosome IV and XII (*CEN4* and *CEN12*), the subtelomeric region on the right arm of chromosome VI (*TEL6R*), and a condensin-depleted region in the *SEC3* gene on chromosome V (*SEC3*) was quantified by qPCR. Data was normalized to Brn1 enrichment at the *CEN4* locus in *YCG1 BRN1-3V5* cells in each experiment and represent mean values of 4 biological replicates +/- 1 standard deviation. Significance was determined by an unpaired t-test (*p<0.05). **(B)** ChIP-qPCR as in (A), except that strains were arrested in G1 with alpha-factor or in metaphase with nocodazole, prior to processing. Data was normalized to Brn1 enrichment at the *CEN4* locus in *YCG1 BRN1-3V5* cells arrested in mitosis in each experiment, and represent mean values of 3 biological replicates +/- 1 standard deviation. Significance was determined by an unpaired t-test (*p<0.05). For centromeric loci that did not show statistically significant increases in binding, the p-values are indicated. Flow cytometry plots confirming cell-cycle positions for all experiments are shown.

Methods

Yeast Strains

A complete list of strains used in this study can be found in S1 Table. All experiments were performed at 30°C, unless otherwise indicated. Strains were grown in rich medium with 2% dextrose, except for strains harboring *MET3p-CDC20*, which were grown in synthetic complete medium lacking methionine with 2% dextrose.

Epitope-tagging of genes was achieved by integrating 3HA-His3MX6, 3V5-kanMX6, or 13Myc-His3MX6 in place of the stop codon at the genomic locus of each gene, as indicated in S1 Table. To generate strains that could be synchronized in metaphase, the methionine-regulatable *MET3* promoter was integrated upstream of *CDC20* using plasmid pBO1105. pBO1105 is a modification of Ylp22(*TRP1*) *MET3p-CDC20* (Uhlmann 2000) in which the Ylp22 vector has been replaced with pAG25 (J.J. Li, personal communication). Where indicated, the *TEF1* promoter was integrated upstream of the start codons of condensin subunits, as previously described (Benanti 2007). Mutations in *YCG1* were introduced into the genome by deleting the non-essential 3' end of the gene, followed by integration of PCR products that replace the 3' sequence and include the indicated mutations. All mutations were confirmed by sequencing. For proteasome inhibition experiments, Ycg1 was tagged in strain YUS5, which carries mutations that increase its sensitivity to proteasome inhibitors (Heinemeyer 1997, Howard 2012).

To assay silencing and recombination at the rDNA locus, *ycg1-K977A* and *TEFp-YCG1* were integrated into strain JS306 and strains were assayed as previously described (Smith 1999). To integrate an extra copy of *YCG1* at the *URA3* locus, *YCG1* (with 362 base pairs of its upstream sequence) was cloned into pRS306 and the resulting vector was digested with NcoI for integration at *URA3*. Single copy integration was confirmed by PCR. Strains expressing temperature-sensitive condensin alleles were previously described in (Li 2011).

Cycloheximide-chase assays

To assay protein degradation, cycloheximide (50 μg/mL) was added to cells and samples taken after the indicated number of minutes. At each time point equivalent optical densities of cells were collected. To assay stability upon proteasome inhibition, cells were grown in synthetic complete medium lacking proline with 0.003% SDS and 2% dextrose, then treated with DMSO or 5 μg/ml MG132 for 2 hours prior to the addition of cycloheximide. Where indicated, cells were arrested with 10 μg/ml alpha-factor for 2.5 hours, or 10 μg/ml nocodazole for 2 hours, before the addition of cycloheximide. In all experiments cell-cycle arrest was verified by flow cytometry.

Western blotting

Samples were prepared for Western Blotting by resuspending equivalent optical densities of cells in preheated SDS sample buffer (50 mM Tris pH 7.5, 5

mM EDTA, 5% SDS, 10% glycerol, 0.5% β-mercaptoethanol, bromophenol blue, 1 µg/ml leupeptin, 1 µg/ml bestatin, 1 mM benzamidine, 1 µg/ml pepstatin A, 17 µg/ml PMSF, 5 mM sodium fluoride, 80 mM β-glycerophosphate and 1 mM sodium orthovanadate), followed by incubation at 95°C for 5 minutes. Glass beads were then added and samples were bead beat using a Biospec Mini-Beadbeater for 3 minutes. Samples were clarified by centrifugation and analyzed by SDS-PAGE followed by Western blotting. Western blots were carried out with antibodies against GFP (clone JL-8, Clontech), Clb2 (y-180, Santa Cruz Biotechnology), Cdc28/Cdk1 (yC-20, Santa Cruz Biotechnology), HA (clone 12CA5), V5 (ThermoFisher), Myc (clone 9E10, Covance), and G6PDH (Sigma). Where indicated, quantitation was performed using a BioRad ChemiDoc Touch imaging system and the accompanying ImageLab software.

Cell-cycle arrest

G1 cell-cycle arrest was achieved by incubating logarithmic-phase cells with 10 µg/ml alpha-factor for 2-3 hours, as indicated. Mitotic arrest was achieved by treating cells with 10 or 20 µg/ml nocodazole for 2-3 hours, or by adding 5X L-methionine (0.1 mg/L final concentration) to *MET3p-CDC20* strains (growing in medium without methionine) for 3.5 hours. Where indicated *MET3p-CDC20* strains were arrested in mitosis as above, then released into medium without methionine containing alpha-factor for 2.5 hours to synchronize cells in G1, followed by release

into medium without methionine or alpha-factor. Details of specific arrest-release experiments are indicated in figure legends.

Cell cycle analysis

Cell-cycle positions were confirmed by flow cytometry. Cells were fixed and labeled with Sytox Green (Invitrogen) as previously described (Landry 2012). Samples were analyzed using a FACScan (Becton Dickinson) and data analyzed with FlowJo (Tree Star, Inc.) software. Where indicated, fixed cells were sonicated and percentage of budded cells determined by counting at least 100 cells/sample.

rDNA Silencing and Stability Assays

rDNA silencing and stability were assayed in strains derived from JS306, as previously described (Smith 1999). In these strains, two PolII-regulated marker cassettes are integrated into different rDNA repeats: a single *MET15* reporter gene (embedded in a Ty1 element) is integrated within NTS2 of one rDNA repeat, and a *mURA3/HIS3* expression cassette is integrated within the 18S rRNA-coding region of a second repeat.

In this assay, the *MET15* reporter is used to score an increase in recombination between rDNA repeats. The expression of *MET15* results in white colonies on MLA plates (Pb+ plates), loss of the *MET15* gene results in dark brown colonies or sectors (as seen in the *sir2Δ* strain), and if the *MET15* gene is present, but is silenced, the colonies are a tan color. Strains are scored as having increased

recombination between rDNA repeats if dark brown and sectored colonies are observed on MLA plates, which indicates loss of the *MET15* gene. Although a tan color indicates *MET15* gene is present, but silenced, the shade of tan is variable between experiments and therefore not used to infer the degree of silencing.

In the same strains the *mURA3/HIS3* reporter is used to assay silencing. Strains that are capable of silencing do not express *mURA3* and thus can't grow on –Ura plates, however *HIS3* is incompletely silenced so strains still grow on –His plates. For this reason, growth on –His is used as a confirmation that the strains retain the *mURA3/HIS3* cassette. Strains that grow similarly on –His and –Ura plates are scored as having a loss of silencing of the rDNA locus. *sir2Δ* mutants were previously shown to have both decreased silencing and increased recombination (Smith 1999), and serve as a positive control for both readouts.

Co-immunoprecipitation Assays

Cell pellets from 30 optical densities of arrested cells were lysed by resuspension in HEPES lysis buffer (25mM HEPES-OH pH 7.5, 250mM NaCl, 0.2% Triton, 1mM EDTA, 10% glycerol, 1 µg/ml leupeptin, 1 µg/ml bestatin, 1 mM benzamidine, 1 µg/ml pepstatin A, 17 µg/ml PMSF, 5 mM sodium fluoride, 80 mM β-glycerophosphate and 1 mM sodium orthovanadate), followed by 3 cycles of bead-beating for one minute each (with 5 minute incubations on ice between cycles). Protein concentrations were measured by Bradford assay and equal amounts of total protein were incubated with 2µL mouse anti-V5 antibody (ThermoFisher) for 3 hours, followed by addition of 25µL protein G magnetic beads

(NEB) for 1 hour. Beads were washed 3X with HEPES lysis buffer and proteins were eluted by boiling in 2X sample buffer.

Doubling time analysis

Cultures were grown to logarithmic phase, then diluted to 0.1 optical densities and 100µL of each was added in triplicate to a round bottom 96-well plate. Cell proliferation was monitored by growing cultures at 30°C with shaking in a Tecan Infinite M200 Pro plate reader and measuring optical density at 600nM every 20 minutes until cultures reached approximately 0.8 OD. Doubling times were calculated by fitting data points between 0.15 OD and 0.6 OD to an exponential growth equation using GraphPad Prism software.

Chromosome spreads

Chromosome spreads to analyze condensin association with chromatin and rDNA morphology were performed as previously described (Lavoie 2002, D'Ambrosio 2008, Cuylen 2011). 3HA-tagged Ycg1 was detected with mouse anti-HA antibody (clone 12CA5), 3V5-tagged Brn1 and 3V5-tagged Net1 were detected with mouse anti-V5 antibody (ThermoFisher), all in combination with Alexa Fluor 488-conjugated goat anti-mouse IgG (ThermoFisher) and DAPI. A wild-type strain lacking both epitope tags was used as a negative control in all experiments. To quantify Ycg1 and Brn1 chromatin binding, Alexa Fluor 488 fluorescence intensities within an area encompassing the merged Alexa Fluor and DAPI images

were measured after background subtraction in ImageJ software. At least 190 cells were quantified for each sample, in each experiment. To score condensation of the rDNA, the rDNA structure (evident both by Net1 staining and the conformation of the DAPI-stained nucleolar DNA) in at least 200 cells were classified as either puffs (decondensed) or loop/lines (condensed), as previously described (Guacci 1997, Lavoie 2002, Lavoie 2004). For all chromosome spreads performed on synchronized cultures, cells were first arrested with 10µg/ml alpha-factor or 20µg/ml nocodazole for 3 hours.

Chromosome immunoprecipitation

Chromosome immunoprecipitation (ChIP) was performed as previously described (Leonard 2015) with the following modifications. For asynchronous and nocodazole arrested cultures, 40 optical densities (ODs) of each culture were lysed in a Mini-Beadbeater (Biospec) and lysates were sonicated using a Diagenode Biorupter. For alpha-factor arrested cultures, 70 OD were used. Brn1-3V5 was immunoprecipitated with mouse anti-V5 (ThermoFisher) coupled to Protein G magnetic beads (New England Biolabs). Eluted DNA was quantified by qPCR on an Eppendorf Realplex system. Primers used for qPCR are listed in S2 Table.

Table 2.1. Strains list.

Name	Genotype	Background
YBL320	*MATa his3Δ leu2Δ met15Δ ura3Δ YHP1-13Myc-LEU2 YOX1-3V5-KanMx TOS4-3FLAG-Hyg HCM1-3HA-HIS3*	S288C
YCG1-GFP	*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 YCG1-GFP-HIS3*	S288C
YTD33	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 YCG1-3HA-HIS3*	W303
YTD82	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 BRN1-3HA-HIS3*	W303
YTD83	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 YCS4-3HA-HIS3*	W303
YTD84	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 SMC2-3HA-HIS3*	W303
YTD80	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 SMC4-3HA-HIS3*	W303
YUS5	*MATa leu2-3, 112 ura3, his3-11,15 CanS GAL2 pup1-T30A pre3-T20A*	W303
YTD43	*MATa leu2-3, 112 ura3, his3-11,15 CanS GAL2 pup1-T30A pre3-T20A YCG1-3HA-HIS3*	W303
YTD36	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ycg1Δ973-1035-3HA-HIS3*	W303
YTD184	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1Δ986-1035-3HA-HIS3*	W303
YTD128	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1Δ973-985-3HA-HIS3*	W303
YTD148	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-K977A-3HA-HIS3*	W303
YTD200	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-R976A-3HA-HIS3*	W303
YTD201	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-R978A-3HA-HIS3*	W303
YTD164	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-R980A-3HA-HIS3*	W303
YTD336	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TEF1p-YCG1-3HA-HIS3*	W303
YTD337	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TEF1p-BRN1-3HA-HIS3*	W303
YTD353	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TEF1p-YCS4-3HA-HIS3*	W303

YTD349	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TEF1p-SMC2-3HA-HIS3*	W303
YTD362	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TEF1p-SMC4-3HA-HIS3*	W303
YTD276	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 YCG1-3HA-HIS3 NatMX6-MET3p-3HA-CDC20*	W303
YTD361	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TEF1p-YCG1-3HA-HIS3 NatMX6-MET3p-3HA-CDC20*	W303
YTD290	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-K977A-3HA-HIS3 NatMX6-MET3p-3HA-CDC20*	W303
YTD302	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Ycg1-3HA-HIS3 BRN1-3V5-kanMX6 NatMX6-MET3p-3HA-CDC20*	W303
YTD355	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TEF1p-YCG1-3HA-HIS3 BRN1-3V5-kanMX6 NatMX6-MET3p-3HA-CDC20*	W303
YTD394	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Ycg1-3HA-HIS3 YCS4-3V5-kanMX6 NatMX6-MET3p-3HA-CDC20*	W303
YTD395	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TEF1p-YCG1-3HA-HIS3 YCS4-3V5-kanMX6 NatMX6-MET3p-3HA-CDC20*	W303
YTD396	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Ycg1-3HA-HIS3 SMC2-3V5-kanMX6 NatMX6-MET3p-3HA-CDC20*	W303
YTD397	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TE1Fp-YCG1-3HA-HIS3 SMC2-3V5-kanMX6 NatMX6-MET3p-3HA-CDC20*	W303
YTD297	*MATa ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 YCG1-3HA-HIS3 BRN1-3V5-KAN*	W303
YTD342	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TEFp-YCG1-HA-HIS3 BRN1-V5-KanMx6*	W303
YTD199	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-S973A S975A T979A S981A-3HA-HIS3*	W303
YTD176	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-E982A E984A-3HA-HIS3*	W303
YTD284	*MATa/MATα ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 can1/can1 YCS4-13Myc-HIS3/YCS4*	W303

YTD268	*MATa/MATα ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 can1/can1 YCG1-3V5-KanMX6/YCG1 YCS4-13Myc-HIS3/YCS4*	W303
YTD269	*MATa/MATα ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 can1/can1 ycg1-K977A-3V5-KanMX6/YCG1 YCS4-13Myc-HIS3/YCS4*	W303
YTD274	*MATa/MATα ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 can1/can1 SMC2-13Myc-HIS3/SMC2*	W303
YTD285	*MATa/MATα ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 can1/can1 YCG1-3V5-KanMX6/YCG1 SMC2-13Myc-HIS3/SMC2*	W303
YTD267	*MATa/MATα ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 can1/can1 ycg1-K977A-3V5-KanMX6/YCG1 SMC2-13Myc-HIS3/SMC2*	W303
YTD275	*MATa/MATα ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 can1/can1 SMC4-13Myc-HIS3/SMC4*	W303
YTD286	*MATa/MATα ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 can1/can1 YCG1-3V5-KanMX6/YCG1 SMC4-13Myc-HIS3/SMC4*	W303
YTD255	*MATa/MATα ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 can1/can1 ycg1-K977A-3V5-KanMX6 SMC4-13Myc-HIS3*	W303
JS306	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3*	
JS576	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 sir2Δ::KanMX4*	
YHA212	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 YCG1-3HA-HIS3*	
YHA214	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 ycg1-K977A-3HA-HIS3*	
YHA215	*MATa his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1::Ty1-MET15, mURA3/HIS3 Hyg-TEF1p-YCG1-3HA-HIS3*	
YTD430	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 can1-100 ycg1-K977A-3HA-HIS3 URA3::YCG1-3HA*	W303
MW836a	*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*	S288C
Y10100	*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 ycg1-2::KanMX6*	S288C
Y9804	*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 brn1-9::KanMX6*	S288C
YJB653	*MATa ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 YCG1-3HA-HIS3 NET1-3V5-KAN*	W303

YJB651	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TEFp-YCG1-HA-HIS3 NET1-V5-KanMx6*	W303

Table 2.2. qPCR Primers

Primer	Location	Sequence	Reference
CEN5DZ-F	Condensin-depleted region in *SEC3* on Chr V	TTTCCTCTCCTCCTGCTTTATTC	this study
CEN5DZ-R	Condensin-depleted region in *SEC3* on Chr V	GAGGCTGAACTGAGACGATTAG	this study
rDNA9-F	rDNA, intergenic spacer 1	AGCCTACTCGAATTCGTTTCC	Clemente-Blanco et al, *Nat Cell Biol*, 2011
rDNA9-R	rDNA, intergenic spacer 1	ATAGTGAGGAACTGGGTTACC	Clemente-Blanco et al, *Nat Cell Biol*, 2011
CEN4-F	Chr IV centromere	CCGAGGCTTTCATAGCTTA	Bizzari and Marston, *J Cell Biol*, 2011
CEN4-R	Chr IV centromere	ACCGGAAGGAAGAATAAGAA	Bizzari and Marston, *J Cell Biol*, 2011
CEN12-F	Chr XII centromere	CTTTACGCGGGTGTGTACTT	this study
CEN12-R	Chr XII centromere	CAACCAAACTGGTGTATGCTAATATC	this study
Tel0.6-F	Chr VI telomere, right arm	CAGGCAGTCCTTTCTATTTC	Hoppe et al, *Mol Cell Biol*, 2002
Tel0.6-R	Chr VI telomere, right arm	GCTTGTTAACTCTCCGACAG	Hoppe et al, *Mol Cell Biol*, 2002

CHAPTER III

Discussion

Cell cycle dependent changes in chromosome conformation were initially characterized in the 1880s by Walther Flemming. These early observations described a major decrease in chromosomal volume during a phase of cellular growth, which was named mitosis. Since then, this decrease in chromosomal volume during mitosis has been described as condensation, and is set in contrast to higher volume interphase chromosome structure. Condensation is an active process, by which many proteins are loaded onto chromatin and/or activated to structure the genome in preparation for mitotic segregation (Koshland 1996, Nasmyth 2002). A key player in this process is the conserved eukaryotic condensin complex.

The condensin complex is a five-subunit protein complex that is essential for mitotic condensation and segregation in eukaryotes (Hirano 1997, Hirano 2012). It was discovered in 1997 by Hirano et al. as a factor necessary for condensation in *Xenopus laevis*, and has since been identified in eukaryotic organisms from yeast to mammals (Hirano 1997, Sutani 1999, Schmiesing 2000, Freeman 2000). Since its initial discovery, the repertoire of known condensin functions has been expanded to include functions outside of mitosis, such as gene dosage regulation, resolution of replicated DNA, and gene clustering (D'Ambrosio 2008, Haeusler 2008, Johzuka 2009, Hirano 2016). Condensin inactivation in

budding yeast and mammalian cell lines suggests that condensin function during interphase is not essential for viability, but that mitotic condensin function is (Lavoie 2002, Hirano 2016).

Condensin appears to carry out its interphase and mitotic functions via positive supercoiling of DNA. How can the same enzymatic activity cluster a handful of loci during interphase and condense entire chromosomes during mitosis? At least two mechanisms of condensin regulation help to explain this. First, condensin-mediated supercoiling occurs at a basal level during interphase, and is hyperactivated by kinases during mitosis (Takemoto 2004, St. Pierre 2009). This difference in activation likely helps to distinguish between clustering during interphase and condensation during mitosis. Second, condensin function is regulated by its localization on chromatin. This regulated recruitment vastly enriches condensin at a small number of specific sites during mitosis to facilitate biorientation and chromosome arm segregation (Verzijlbergen 2014, Johzuka 2006). In contrast, interphase condensin appears to be more dispersed globally (Leonard 2015), which, in addition to being less enzymatically active (Takemoto 2004, St. Pierre 2009), likely decreases condensin dependent genome organization.

Rate Limiting Subunit Levels Limit Condensin Formation/Activity

Here, we describe a new mechanism that modulates condensin levels in order to regulate condensin activity in budding yeast. This mechanism limits the

expression of a single condensin subunit, Ycg1, during the cell cycle via cyclical transcription and constant proteasomal turnover. We show that cell cycle dependent Ycg1 expression limits condensin formation (Figure 2.1 and 2.11), with less condensin in G1, when Ycg1 levels are low, and more in mitosis when Ycg1 levels are high. Interestingly, stabilizing Ycg1 or increasing its transcription was sufficient to increase total condensin levels in G1. Further, increased condensin complex levels caused increased condensin association with DNA in G1 (Figure 2.12), which correlate with increased G1 duration (Figure 2.7), leading to a decrease in proliferation (Figure 2.5). These observations are the first to show that condensin complex levels change with the cell cycle in any organism. This newly described mechanism, along with cell cycle specific activation and condensin loading, enables eukaryotes to rapidly switch between basal activity (interphase), and hyperactivity (mitosis). Figure 3.1

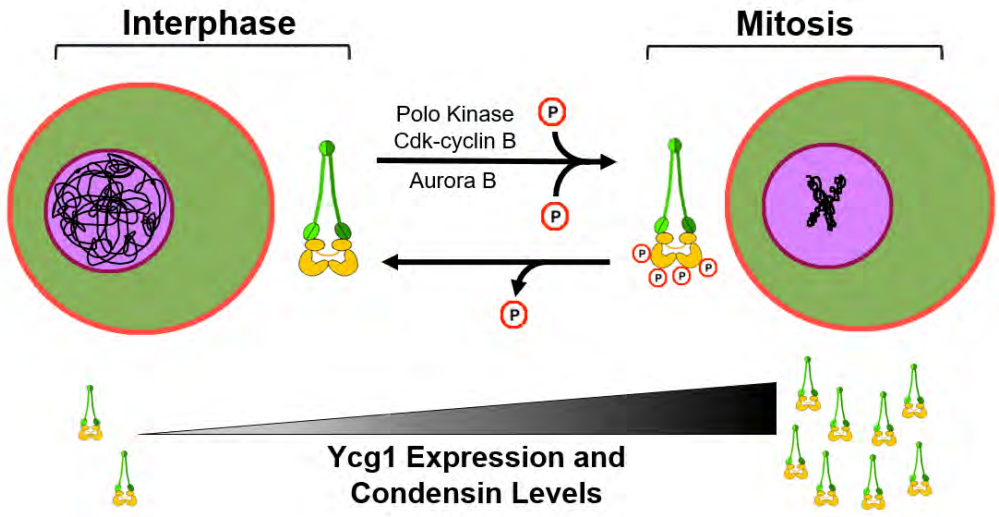


Figure 3.1: Condensin Regulation in Relation to the Cell Cycle. A cartoon showing the known mechanisms of condensin regulation. Polo Kinase and Cdk-cyclin B phosphorylation of during early mitosis activates condensin. Aurora B phosphorylation, and other mechanisms, recruit condensin to pericentric regions during early mitosis, and chromosome arms during late mitosis. Ycg1 expression is low in interphase, which limits condensin formation (left), while expression increases during the cell cycle, leading to increased Ycg1 and condensin levels during mitosis (right). Together, these mechanisms allow for clustering of specific loci during interphase, and large scale condensation during mitosis.

Factors That Influence Ycg1 Turnover

Further insights into the mechanism of Ycg1 expression regulation is needed to round out the current model. Two immediate questions present themselves; 1) what factors influence Ycg1 turnover, and 2) what mechanisms regulate *YCG1* transcription? Answering these two questions should increase the understanding of yeast condensin regulation, and may provide insight into condensin regulation in other eukaryotes.

Future experiments to identify modulators of Ycg1 stability should benefit from the data presented here. Indeed, many UPS modulators were screened and are presented in Appendix A. This screen is incomplete, and future work should focus on characterizing Ycg1 turnover in the remaining UPS strains which were not covered in the screen. Particularly, the Mot2 and Ste5 E3 ligases, which were not covered in the screen, should be deleted and assayed for Ycg1 turnover. In addition, Rsp5, an essential E3 ligase should also be assayed for effects on Ycg1 turnover using an rsp5 temperature sensitive allele. In the event that no single E3 ligase is found to target Ycg1, crosslinking mass spectrometry might be useful to find factors associated with Ycg1. In the past, this has proven challenging, since E3 ligase/target interactions are transient. Other screening methods may capture these transient interactions and may help identify E3 ligase(s) that target Ycg1. Additional discussion of this topic can be found in Appendix A.

Observations in Chapter II suggest that Ycg1 expression during the cell cycle is strongly influenced by transcription of the *YCG1* gene. Although Ycg1 is unstable, similar turnover rates were observed in the presence of cycloheximide at various cell cycle arrest points (Figure 2.2 and data not shown). This constitutive turnover effectively removes protein stability as a variable, and likely makes *YCG1* transcription the primary determinant of Ycg1 expression during the cell cycle. This tightly controlled transcription-dependent protein expression mechanism might allow Ycg1 levels to be rapidly adjusted in response to cellular cues. In the future, determining which transcription factors control *YCG1* expression in a wildtype

scenario might illuminate potential inputs which alter Ycg1 and thereby, condensin levels. Hcm1, a forkhead transcription factor which drives gene expression in late S-phase is an interesting candidate for *YCG1* regulation. In agreement with this possibility, *YCG1* contains a Hcm1 binding motif (Pramilla 2006), and is expressed most strongly in late interphase (Figure 2.1). Finally, it is worth noting that Brn1 expression decreased by 40% in conjunction with increased condensin levels (Fig 2.11 and Fig 2.12). This, along with condensin's documented impact transcription suggests that the complex may feedback, and regulate the transcription of condensin subunits. Future work is needed to assess whether condensin associates with the *BRN1* locus and whether *BRN1* transcript levels decrease in the *TEF-YCG1* background.

Proteasomal Targeting of Ycg1 – Cause or Consequence of Condensin Disassociation from Chromatin?

Condensin functions require interaction with chromatin, which is facilitated by loading mechanisms, such as association with chromatin-associated loading factors. However, the exodus of condensin from one set of loci to another during the cell cycle requires a mechanism for condensin disassociation from chromatin. Recent data from *Xenopus laevis* and budding yeast suggests that condensin's association with chromatin is transient, even when chromosomes are condensed in mitosis (Kinoshita 2015, Robellet 2015). This transient interaction could be a byproduct of condensin activity, or may require additional factors (such as ubiquitin) to unload the complex. In either case, these data suggest that condensin

constitutively disassociates from chromatin, and likely reassociates with loci based on loading factor availability. This mechanism would allow, condensin to rapidly move in response to signals that alter loading factor expression, such as cell cycle position. Might Ycg1 instability either 1) influence condensin unloading from chromatin or 2) limit the nucleoplasmic amount of condensin, thereby limiting subsequent loading?

Ycg1 is found in the chromatin and soluble fractions throughout the cell cycle (Freeman 2000), but where is Ycg1 localized when it is ubiquitinated? To test this directly, Ycg1 could be precipitated from chromatin, cytoplasmic, and nucleoplasmic fractions, followed by western blotting for ubiquitin conjugation to Ycg1. Similarly, the Ycg1 degron region could be localized to the cytoplasm, nucleoplasm, or chromatin via fusion to the appropriate localization signals (Arnold 2014), followed by half-life measurement. These analyses should allow us to determine where in the cell Ycg1 is ubiquitinated and least stable.

In the event that Ycg1 is ubiquitinated on chromatin, Cdc48 may be involved in Ycg1 turnover. Cdc48 is a AAA ATPase that can bind to chromatin-associated ubiquitinated proteins and pulls them off of DNA (Dantuma 2012). If Ycg1 were ubiquitinated on chromatin, it might be pulled off chromatin by Cdc48 (along with condensin disassociation), followed by reloading of condensin with a new Ycg1 subunit. This hypothesis has not yet been tested, but would be a solution for regulating condensin levels, by pulling off and incapacitating excess complex. A simple experiment to test Cdc48's involvement would be to use cdc48 temperature

sensitive alleles, which allow transient inactivation, at which time Ycg1's half-life could be measured.

Together, these analyses should illuminate where Ycg1 ubiquitination and turnover occurs, which should inform if/how Ycg1 turnover regulates condensin's interaction with chromatin.

How Might Aberrant Condensin Loading Delay the Cell Cycle – Segregation, Replication, or Transcription

In Chapter II, we report that increasing Ycg1 expression, by constitutively expressing YCG1, or by interfering with proteasomal targeting causes a delay in progression through the early cell cycle. How increased Ycg1 levels cause this delay is unclear, but may involve increased condensin complex function, which may alter genome segregation, replication, or interphase gene expression. This section focuses on relating the findings of this thesis to known condensin phenotypes, in hopes of informing future experiments.

Condensin is essential for mitotic segregation of chromatin in budding yeast (Freeman 2000, Lavoie 2002). This is most notably due to its activity at the ribosomal DNA locus, a tandem array of ~150 repeats found on Chromosome XII (Johzuka 2006). In condensin inactivation experiments, Chromosome XII often fails to segregate from the midzone (Freeman 2000). In addition to physically compacting the rDNA, condensin recruits other factors to the locus during anaphase, which stop RNA Polymerase I transcription, which is necessary for segregation (Iacovella 2015). In our experiments, condensin levels are increased

throughout the cell cycle in response to constitutive Ycg1 expression (Figure 2.11). However, although we observe a decrease in condensin binding at a region within the rDNA during mitosis (Figure 2.13B), we did not observe changes in mitotic rDNA structure (Figure 2.9). In addition, we probed for changes in rDNA silencing and rDNA stability and found no affect upon constitutive Ycg1 expression (Figure 2.6). Finally, arrest/release experiments in the presence on constitutive Ycg1 expression did not delay progression through mitosis (Figure 2.7). Together these results suggest that the condensin mediated delay in cell cycle progression observed in Figure 2.7 is unlikely to result from ribosomal DNA dysfunction.

Though condensin is known to be present on chromatin in eukaryotes during replication, its activity is largely uncharacterized. Here we show that condensin levels are relatively low in early interphase (G1), and high during mitosis (Figure 2.11). We would anticipate intermediate condensin levels during S-phase based on the corresponding Ycg1 expression level (Figure 1.1). However, this was not assessed, and future work should characterize the condensin complex levels during S-phase by Co-Immunoprecipitation. If condensin levels are increased upon constitutive Ycg1 expression, then it may alter ssDNA annealing or supercoiling as it has been shown to do at termination sites of active genes (Sutani 2015), and thereby affect replication. Importantly, these possibilities are speculative, and may not explain the proliferation defects observed in the *TEFp-YCG1* and *ycg1-K977A* backgrounds. Notably, the cell cycle delay observed involves progression through the G1/S transition, which occurs prior to DNA

synthesis (Figure 2.7). Nonetheless, the increased condensin formation in the strains described here might be useful to assess condensin activity during S-phase.

Condensin has been implicated in regulating gene expression, both positively and negatively, likely by changing chromatin structure. Might increasing condensin levels alter gene expression in the early cell cycle, and thereby delay the G1/S transition? In budding yeast, condensin silences the mating type locus (Bhalla 2002), RNA Polymerase II promoters proximal to tRNA genes (Haeusler 2008), and telomere proximal genes (Machin 2004). Conversely, it is possible that condensin may increase expression of specific loci, as has been shown in mammalian cells (Li 2015). In the future, condensin's effect on interphase gene expression should be measured by RNA-seq of constitutive Ycg1 expression strains at various points in the cell cycle. This analysis would identify which, if any, of the factors necessary for G1/S progression are influenced transcriptionally by condensin levels. Of particular interest might be *CLN1* and *CLN2* transcription, other SBF/MBF driven genes, or genes involved in cell size, such as ribosomal subunits (Bertoli 2013). These experiments should utilize the *TEFp-YCG1* strains, or a newly generated strain with closer to wildtype expression (Appendix B), and not the *ycg1-K977A* strain, which may inactivate some condensin functions.

Where Might Condensin Load During Interphase Upon Ycg1 Overexpression?

Data presented in Figure 2.12 suggest that increasing condensin levels increases condensin loading during G1, and slows progression through the G1/S transition (Figure 2.7). However, thus far tRNA genes and a few other G1 loading sites have been assessed and exhibit unaltered condensin binding in the presence of Ycg1 overexpression (data not shown). This suggests that increasing condensin levels may not increase TFIIIC dependent loading onto chromatin during interphase.

Condensin binding to nucleosome depleted regions (NDRs) might explain the discrepancy between increased G1 loading (Fig 2.12) and the lack of enrichment at known G1 binding sites (Fig 2.13) (Piazza 2014, Leonard 2015). Future experiments should seek to probe this relationship by analyzing condensin overexpression ChIP-seq binding versus nucleosome occupancy in G1 arrested cells (Piazza 2014). Further, if this analysis shows increased condensin enrichment at NDRs in the *TEFp-YCG1* background, then a cause-effect relationship could be assayed using an inducible NDR (Adkins 2006). Regardless of whether condensin localizes to NDRs, its genome-wide binding pattern may provide clues as to how increased condensin loading affects interphase chromatin. Of particular interest are SBF and MBF binding sites, which associate with and are repressed by Whi5 in budding yeast prior to START. Previous reports have shown the retinoblastoma protein (analogous to Whi5) can recruit condensin to genomic loci in *Drosophila* and mammalian cell lines during interphase (Longworth 2008, Coschi 2014). If Whi5 recruits condensin to chromatin in yeast, it may do so at

SBF/MBF genes which might cause transcriptional changes, thus influencing START.

Measuring Changes in Interphase Chromatin Conformation – Interchromosomal or Intrachromosomal Condensin Activity?

Data presented in Figure 2.11 shows that condensin levels increase upon Ycg1 expression, and Figure 2.12 shows that this extra condensin is loaded onto chromatin. However, at present, no phenotypic data has been collected which shows that this extra condensin alters the conformation of the genome. This section introduces methods which may prove useful in measuring potential changes in condensin activity.

Condensin has been shown to cluster many of the 274 tRNA genes (tDNA), which are dispersed throughout the genome, to the perinucleolus during interphase (D'Ambrosio 2008, Haeusler 2008). This clustering causes condensin-dependent silencing of tRNA proximal Pol II transcribed genes (Haeusler 2008). Interestingly, this interchromosomal clustering function of condensin is interphase specific, and may cause inappropriate clustering of newly created condensin hotspots in the *TEFp-YCG1* strain background. ChIP-seq results from G1 arrested strains constitutively expressing Ycg1 would illuminate these new condensin hotspots, if they exist. DNA FISH could be used to determine whether these new loci cluster to the perinucleolus/tDNA, and RT-qPCR could be used to test for changes in hot-spot proximal gene expression.

Intrachromosomal condensation occurs at a high level upon activation of condensin's supercoiling activity in mitosis. Although this enzymatic activity is less prevalent during interphase, increasing condensin levels may lead to measurable chromosome condensation. We tested this by visualizing the budding yeast ribosomal DNA locus (Figure 2.9), a ~150 repeat locus that lies on the right arm of chromosome XII (Johzuka 2006). This region is structured by condensin throughout the cell cycle, with visual intermediates during the process of chromosome condensation (Robellet 2015). We found that rDNA conformation is unaffected upon constitutive expression of Ycg1 (Figure 2.9). These data suggest that the increased condensin observed upon overexpression does not affect the structure of the rDNA locus during interphase. However, it remains possible that condensin is acting intrachromosomally during interphase at other loci. Indeed, tools are readily available to assess the condensation status of chromosome arms and should be utilized to test this possibility. Specifically, strains with two sets of LacO repeats on a chromosome arm have been established, and in the presence of LacI-GFP, allow for assessment of intrachromosomal condensation (Vas 2006, D'Ambrosio 2008). Assessing possible changes in chromosome arm condensation in the wildtype, *TEFp-YCG1*, and *ycg1-K977A* backgrounds during interphase should elucidate whether intrachromosomal condensation occurs in the presence of increased condensin formation.

The gold standards for measuring changes in chromosome conformation are Hi-C and related techniques. These techniques utilize crosslinking, followed by

restriction digestion and re-ligation, which creates “chimeras” which are comprised of two interacting strands of DNA (Belton 2012). These chimeras are purified, then sequenced to deconvolute a genome-wide interaction map (Belton 2012). This type of analysis would help determine whether increased condensin levels alter the conformation of the genome. These experiments might be particularly interesting in strains arrested in early interphase, to correct for changes in cell cycle distribution upon constitutive Ycg1 expression. Most notably, Hi-C can identify interactions which occur inter or intrachromosomally, thus addressing both possibilities discussed above.

Could Ycg1 Act Independent of Condensin to Delay the Cell Cycle?

Though we observed increased condensin levels in the Ycg1 overexpression background, it is possible that the proliferation phenotypes we observed are due to excess non-condensin associated Ycg1. Though Ycg1 has not been characterized to act outside of condensin, the possibility has not been thoroughly tested within this project or in published reports. Two plausible possibilities are; 1) Ycg1 aggregates when expression is increased, causing cellular dysfunction, and 2) Ycg1 can associate with chromatin independent of the condensin complex and upon doing so, interferes with transcription or replication of DNA.

A correlation between overexpression and protein aggregation has been observed (Fink 1998), and should be considered in the case of Ycg1. Indeed,

protein aggregates can cause cellular dysfunction (Bence 2001), which might explain the proliferation defect in the presence of increased Ycg1 expression. In Chapter II, we tagged Ycg1 with Green Fluorescent Protein (GFP) to track protein levels during the cell cycle (Figure 1.1). Indeed, these strains could be used to track Ycg1 localization upon constitutive expression. If Ycg1-GFP forms puncta, this could be a sign of aggregation, which could cause the observed proliferation defect. Future work should attempt to recapitulate the G1/S delay phenotype with closer to wildtype expression of Ycg1. Data in Appendix B shows a viable method that might be useful in achieving constitutive expression at closer to wildtype asynchronous protein levels. Strains expressing Ycg1 constitutively at nearer to wildtype levels should be assayed for proliferation defects, to separate vast Ycg1 overexpression (and potential aggregation) from increased interphase condensin levels.

Ycg1 is necessary for the efficient association of condensin with DNA *in vitro* and *in vivo* (Piazza 2014). This association relies on Ycg1's HEAT repeat domain, which interacts with DNA directly (Piazza 2014). This direct interaction with DNA raises the possibility that upon overexpression, Ycg1 could associate with chromatin independently of the condensin complex. Previous work has shown that condensin subunits disassociate from the rDNA in mitotic arrested yeast upon inactivation of any single condensin subunit, suggesting that condensin subunits associate weakly, if at all, with DNA outside of the *bona fide* complex (Lavoie 2002). However, increasing Ycg1 protein levels by overexpression might

exacerbate an otherwise weak interaction with chromatin. In this case, Ycg1 might interfere with proliferation by associating with DNA and competing with transcription or replication factors. To address this, the association of Ycg1 with chromatin should be measured by cellular fractionation. If increased Ycg1 expression increases Ycg1 association with the chromatin fraction more than other condensin subunits, then Ycg1 may interact with chromatin without condensin. This could be further tested using a temperature sensitive allele of a non-Ycg1 condensin subunit, to inactivate condensin, followed by measurement of Ycg1 levels in the chromatin fraction. If Ycg1 continues to associate with chromatin after condensin inactivation, then Ycg1 likely associates with chromatin without condensin. Indeed, G1 arrest/release experiments in the absence of condensin function could determine whether increased Ycg1 causes the G1/S delay observed.

Condensin Subunit Levels in Multicellular Eukaryotes: Conservation of Rate Limiting Subunit Regulation?

Recent reports suggest that condensin subunit Cap-H2 is unstable in *Drosophila melanogaster* (Buster 2013, Nguyen 2015). Furthermore, increasing expression of this subunit by overexpression or degron mutation leads to a visible increase in clustering of interphase chromatin (Buster 2013). This intermediate state looks like partial condensation and is called a “chromatin gumball” phenotype. This phenotype suggests that Cap-H2, the Kleisin subunit of condensin II in flies may be rate limiting for condensin formation, since increasing its

expression alone increases interphase clustering (Buster 2013, Nguyen 2015). Conversely, condensin II knockdown in flies and mammalian cells results in chromatin swelling and abnormal nuclear shapes (George 2014). The general findings described in these reports, along with data presented here, suggest that the regulation of condensin by rate limiting subunit expression may be present throughout eukaryotes, and may be important for structuring the interphase nucleus.

Notably, the chromatin gumball phenotype described in Buster 2013 was not observed upon constitutive Ycg1 expression in budding yeast. This may not be surprising, as condensation of the budding yeast genome is difficult to observe, due to its small genome size (12.5Mbp) and relatively low total mitotic condensation (~2-fold) (Guacci 1994). Additionally, neither the closest ortholog to the Cap-H2 targeting F-box protein from Buster et al. (Met30), nor total F-box inactivation increased Ycg1 stability in budding yeast (Appendix A) (Buster 2013). This suggests that the E3 ligase involved in Cap-H2 proteasomal targeting in flies is not sufficient for Ycg1 turnover in yeast.

In addition to flies and yeast, rate limiting subunit expression may regulate condensin in mammals. Xu et al. showed that chromatin becomes more compact and less transcriptionally active during maturation of the erythroid lineage in a condensin II-dependent manner (Xu 2006). Further analysis found that overexpression of condensin II subunit Cap-G2 was sufficient to reduce gene expression associated with maturation and to differentiate transformed cells of this

lineage (Xu 2006). These data suggest that increasing expression of one condensin subunit, Cap-G2, is sufficient to alter gene expression in erythroid cell types, potentially by increasing interphase chromatin compaction. The proliferation phenotype upon Cap-G2 expression correlates with our observation that increased Ycg1 levels slow proliferation (Figure 2.7), and the increased chromatin compaction phenotype is similar to the aforementioned observations from *Drosophila* (Buster 2013). Future experiments are needed to determine whether condensin II is limited by Cap-G2 expression in erythroid cells, or if Cap-G2 acts on its own to repress transcription and compact the genome. In particular, it will be important to determine whether Cap-G2 overexpression causes increases in condensin II formation and association with chromatin.

Assessing the role of limiting condensin subunit expression in other mammalian cell types might best be characterized by tracking condensin subunit protein levels over time in the presence of cycloheximide. This may reveal which subunit is limiting, as instability has been noted in the rate limiting subunits in budding yeast (Figure 1.1) and *Drosophila* (Buster 2013). Mammalian condensin subunit instability seems plausible, since some high-throughput approaches have identified some condensin subunits as unstable in mammalian cell lines (Yen 2008, Schwanhausser 2011).

Can Rate Limiting Subunit Expression in Yeast Explain Observations in Cancers?

Increased condensin subunit expression is necessary for proliferation in some cancer cell lines (Davalos 2012, Murakami-Tonami 2014, Zhou 2014). However, until recently, increases in condensin levels have been sparsely characterized.

In Chapter II, we observe that condensin levels can be increased by constitutive expression of a single subunit of the complex in budding yeast (Figure 2.11). This observation, along with the aforementioned observations from *Drosophila* (Buster 2013), suggest that condensin levels and function can be influenced by the expression of a single rate-limiting subunit. These observations might explain how the observed increases in one, or a few condensin subunits can cause increased condensin-related functions in cancer cell lines (Davalos 2012, Murakami-Tonami 2014, Zhou 2014).

In addition, the system introduced in Chapter II may help characterize how increased condensin levels alter gene expression, replication, and mitotic segregation, which in turn may provide clues as to how condensin effects cancer cell proliferation. Consider two recent reports, both of which were carried out in cancer cell lines which have higher condensin subunit expression than their non-transformed controls. The first found that colorectal carcinoma cells have increased condensin, which discourages lagging chromosomes during segregation, thus protecting against mitotic catastrophe (Davalos 2012). The second found that neuroblastoma cells have increased condensin that causes altered interphase transcription of DNA damage response genes, thus preventing

apoptosis (Murakami-Tonami 2014). While it is possible that these two condensin-mediated phenotypes (mitotic fidelity and interphase DDR gene expression) are mutually exclusive, it may be more likely that increased condensin levels cause multiple phenotypes during the cell cycle. Deconvolution of these phenotypes from those which are intrinsic to each transformed cell line is a daunting task, thus we propose utilizing the model established in this report. In the future, characterizing the phenotypes associated with increased condensin levels during each cell cycle phase in budding yeast will inform a more complete understanding of condensin biology, which, in turn, may help decipher condensin's role in aiding proliferation in cancer cells.

Appendix A: Screening for Modulators of Ycg1 Stability

Preface

The Ubiquitin-Proteasome System screen described in this section was performed by Kayleigh Gallagher. The *YCG1-V5* plasmid was created by Tyler Doughty. Experiments which follow-up the candidates from the screen, and Cdc53 experiments were performed by TD.

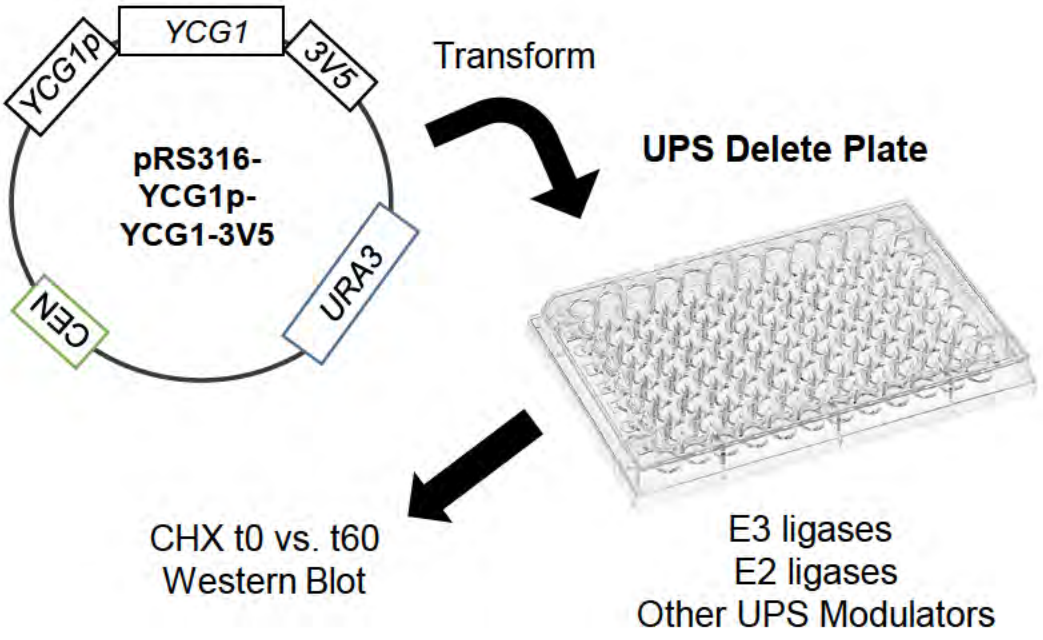
Introduction

Ycg1 expression cycles, with high expression during mitosis and relatively low expression during early interphase (Figure 2.1). This cyclical expression is dictated by cyclical transcription, and constant turnover of Ycg1. Notably, we conclude that Ycg1 turnover depends on the proteasome, since the proteasome-specific inhibitor MG132 is sufficient to stabilize Ycg1 (Figure 2.2). This appendix describes efforts which sought to identify the specific factors necessary for proteasomal targeting of Ycg1.

The eukaryotic proteasome is a large multi-subunit protease which is essential for viability in eukaryotes (Hochstrasser 1996). Turnover by the proteasome is limited by its ability to recognize a protein as a target, which requires polyubiquitination (Hershko 1998). Several classes of modulators regulate target protein ubiquitination, and therefore regulate proteasomal turnover. These factors

include E3 ligases, which conjugate ubiquitin onto proteins, ubiquitin receptors, which associate with the proteasome to help recruit ubiquitinated proteins, and deubiquitinating enzymes, which can counter this process by removing ubiquitin (Hershko 1998). In addition, other factors, such as SUMO ligases can influence proteasomal turnover by changing the affinity of an E3 ligase for its target. Together, these modulators of proteasomal function are collectively called the Ubiquitin-Proteasome System (UPS).

In this appendix, we describe a screen which attempted to identify factors necessary for Ycg1 turnover. Appendix A.1 shows the workflow for the UPS deletion screen. *YCG1p-YCG1-3V5* was cloned onto a single-copy CEN plasmid with the *URA3* selection marker. The 96-well UPS deletion plate containing 96 yeast strains, each with a single UPS modulator deleted (list of strains in Appendix A.2), was transformed with the plasmid above via 96-well transformation (see methods). Each deletion mutant was assessed for changes in Ycg1-V5 level via western blot before and after the addition of cycloheximide. Candidates from this screen were identified as having less Ycg1 turnover than wildtype after cycloheximide treatment, which suggests that the candidate gene influences Ycg1 turnover.



Appendix A.1: Ubiquitin Proteasome Screening for Modulators of Ycg1 Stability. The workflow for the UPS deletion screen is shown. A 96-well plate contains strains which have a single ubiquitin-proteasome system modulator deleted was transformed with pRS316-YCG1p-YCG1-3V5. Ycg1-V5 levels were assessed before and after addition of cycloheximide for 60 minutes.

Results

The UPS deletion screen is shown in Appendix A.2. Some strains did not grow on the UPS deletion plate, and were therefore not assessed (highlighted gray). Other strains, particularly E3-ligase deletion strains, were created to supplement the UPS deletion plate and were assayed similar to the rest of the plate (highlighted orange). Candidates, which increased Ycg1 stability upon deletion, are highlighted in red.

Sample data from the screen is shown in Appendix A.3.A. Three candidates were identified, *pib1*∆*, siz1*∆*,* and *ydr306c*∆*,* based on visual increases in stability after correcting for differences in expression level. *3HA-HIS3* sequence was integrated at the endogenous *YCG1* locus of each candidate strain. Follow-up analysis, which was performed in triplicate, showed that Ycg1 was not more stable in these deletion strains (Appendix A.3.B), which suggests that these candidates were false positives.

Additional E3-ligase screening is shown in Appendix A.4, with focus on inactivation of the Skp-Cullin-F-box ubiquitin ligase complex (SCF) (Bai 1996). The SCF recognizes proteins which interact with the F-box subunit of the complex as targets for ubiquitination (Skowyra 1997). Yeast have 20 F-box proteins, some of which have been shown to have redundant targets (Landry, 2012). Ycg1 stability in the absence of SCF function was important to assess for a few reasons; 1) SCF function might have been required for Ycg1 turnover, 2) Cdc4, an SCF adaptor is

essential, and was not included on the UPS deletion plate, and 3) multiple SCF adaptors may target Ycg1 redundantly, and thus inactivation of the SCF would stabilize Ycg1. Neither dominant negative inhibition or temperature-sensitive inactivation suggest that the SCF is required for Ycg1 turnover (Appendix A.4).

Appendix A.2: Ubiquitin Proteasome Screening for Modulators of Ycg1 Stability.

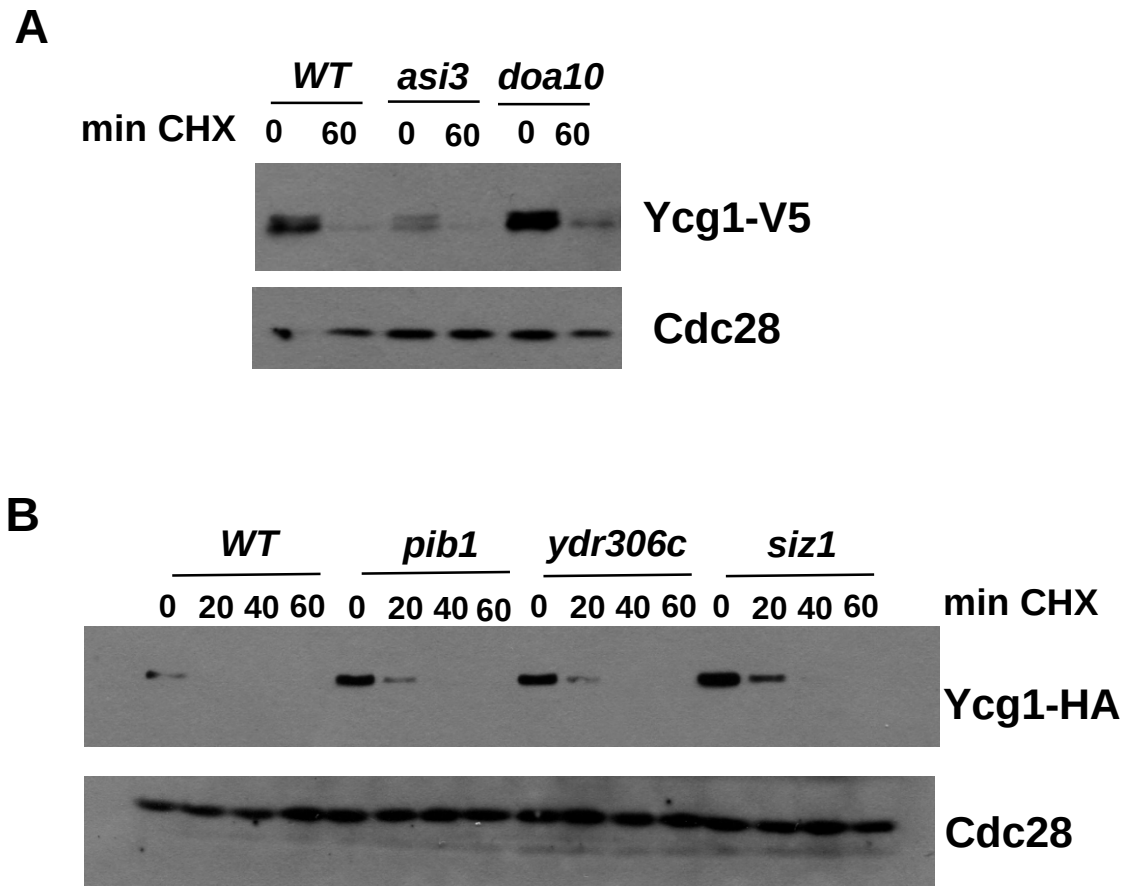
Deletion	Category	Coordinates	Experiment	Results
ubr2	E3	A1	6/30/2014	Unstable
hrt3	E3	A2	7/18/2014	Unstable
ufo1	E3	A3	6/30/2014	Unstable
ubc7	E2	A4	7/23/2014	Unstable
ubp8	DUB	A5	7/30/2014	Unstable
rkr1	E3	A6	7/17/2014	Unstable
dsk2	UB Receptor	A7	7/23/2014	Unstable
ubp15	DUB	A8		
ynl311c	E3	A9	7/17/2014	Unstable
dia2	E3	A10	7/18/2014	Unstable
ubc11	E2	A11	7/7/2014	Unstable
hrd1	E3	A12	7/21/2014	Unstable
lag2	Neddylation	B1	7/30/2014	Unstable
psh1	E3	B2	7/17/2014	Unstable
cos111	E3	B3	7/22/2014	Unstable
ubc5	E2	B4	7/22/2014	Unstable
doa4	DUB	B5	7/8,30/14	Partially Stable
ubc13	E2	B6	7/23/2014	Unstable
ste5	E3	B7		
ydr131c	E3	B8	7/28/2014	Unstable
rub1	Neddylation	B9	7/23/2014	Unstable
san1	E3	B10	7/21/2014	Unstable
siz1	**SUMO E3**	**B11**	**7/18,25/14**	**Partial-8/1/14**
rad23	UB Receptor	B12		
mot2	E3	C1		
pex4	E2	C2	7/23/2014	Unstable
ubr1	E3	C3	7/28/2014	Unstable
otu2	DUB	C4		
dma1	E3	C5	7/18/2014	Unstable
rpn10	UB Receptor	C6	7/7/2014	Unstable
dcn1	Neddylation	C7		
ylr244w	E3	C8	7/21/2014	Unstable

Appendix A.2: Ubiquitin Proteasome Screening for Modulators of Ycg1 Stability. (Continued)

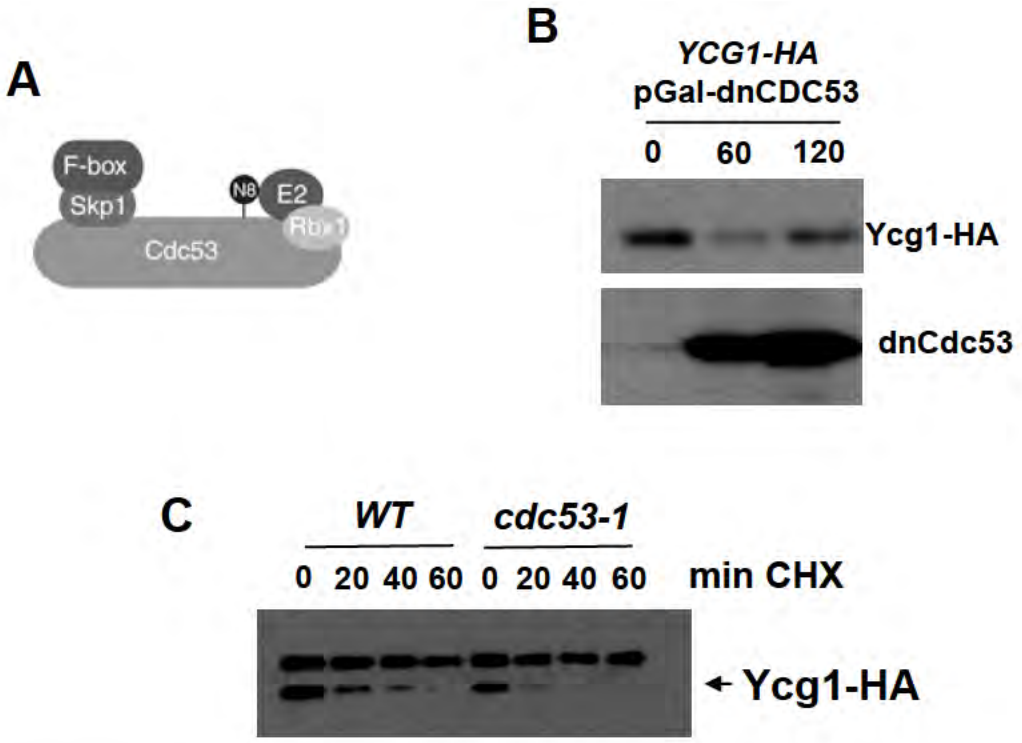
Deletion	Category	Coordinates	Experiment	Results
ufd4	E3	C9	7/21/2014	Unstable
tul1	E3	C10	7/22/2014	Unstable
etp1	E3	C11	7/8/2014	Unstable
ubp2	DUB	C12	7/25/2014	Unstable
nfi1	SUMO E3	D1	7/18/2014	Unstable
uls1	E3	D2	7/21/2014	Unstable
rcy1	E3	D3	7/22/2014	Unstable
ubp12	DUB	D4	7/25/2014	Unstable
yjl149w	E3	D5	7/17/2014	Unstable
ylr352w	E3	D6	7/14/2014	Unstable
mdm30	E3	D7	7/23/2014	Unstable
rpn13	UB Receptor	D8	7/14/2014	Unstable
irc20	E3	D9	7/18/2014	Unstable
ubc12	E2	D10		
ydr219c	E3	D11	7/21/2014	Unstable
cul3	E3	D12	7/21/2014	Unstable
ubp16	DUB	E1	7/25/2014	Unstable
ula1	Neddylation	E2	7/25/2014	Unstable
ubp5	DUB	E3	7/25/2014	Unstable
ama1	E3	E4	8/1/2014	Unstable
ubp13	DUB	E5	7/2/2014	Unstable
cdh1	E3	E6	7/8/2014	Unstable
rad6	E2	E7	7/23/2014	Unstable
ykr017c	E3	E8	7/28/2014	Unstable
rmd5	E3	E9	7/8/2014	Unstable
pex10	E3	E10	7/17/2014	Unstable
ydr266c	E3	E11	7/29/2014	Unstable
ydr306c	**E3**	**E12**	**7/22,28/14**	**Partial-8/1/14**
pib1	**E3**	**F1**	**7/17,25/14**	**Partial-8/1/14**
otu1	DUB	F2	7/2/2014	Unstable
ubp6	DUB	F3	7/30/2014	Unstable

Deletion	Category	Coordinates	Experiment	Results
rtc1	E3	F4	7/28/2014	Unstable
ubp9	DUB	F5	7/30/2014	Unstable
rad5	E3	F6	7/14/2014	Unstable
asi1	E3	F7	7/18/2014	Unstable
uba3	Neddylation	F8	7/25/2014	Unstable
asr1	E3	F9	7/21/2014	Unstable
rtt101	E3	F10	7/18/2014	Unstable
ubc8	E2	F11		
ubp11	DUB	F12	7/29/2014	Unstable
mag2	E3	G1	7/17/2014	Unstable
yuh1	DUB	G2	7/29/2014	Unstable
rad18	E3	G3	7/28/2014	Unstable
hul4	E3	G4	7/21/2014	Unstable
ubp1	DUB	G5	7/30/2014	Unstable
ufd2	E3	G6	8/1/2014	Unstable
asi3	E3	G7	7/18/2014	Unstable
ubp14	DUB	G8		
dma2	E3	G9	7/21/2014	Unstable
ubp7	DUB	G10	7/30/2014	Unstable
saf1	E3	G11	7/18/2014	Unstable
cst9	SUMO E3	G12	7/22/2014	Unstable
ssm4	E3	H1	7/21/2014	Unstable
ubc4	E2	H2	7/23/2014	Unstable
amn1	E3	H3	7/28/2014	Unstable
slx5	E3	H4	7/21/2014	Unstable
tom1	E3	H5	7/7/2014	Unstable
hul5	E3	H6	7/28/2014	Unstable
slx8	E3	H7	7/29/2014	Unstable
ddi1	UB Receptor	H8	7/29/2014	Unstable
ubp3	DUB	H9	7/30/2014	Unstable
bre1	E3	H10	7/22/2014	Unstable
blm10	Other	H11	7/30/2014	Unstable
grr1	E3	H12	7/23/2014	Unstable
met30	E3	Not on plate	7/23/2014	Unstable
pep5	E3	Not on plate	8/4/2014	Unstable
pex2	E3	Not on plate	8/4/2014	Unstable
snt2	E3	Not on plate	8/4/2014	Unstable

Appendix A.2: Ubiquitin Proteasome Screening for Modulators of Ycg1 Stability. A table showing the results of the screen. Gray indicates a strain which was not tested (due to lack of growth). Orange highlighting indicates strains which were established via traditional deletion and tagging methods to supplement the screen. Red indicates strains which were deemed hits.



Appendix A.3: Ubiquitin Proteasome Screening for Modulators of Ycg1 Stability. A) Sample data from the screen is shown. Notably, though protein levels changed in relation to wildtype at t0 in some strains, the stability of Ycg1-3V5 was similar for most strains in this assay. B) As a follow-up to the screen, *YCG1-HA-HIS3* was integrated at the endogenous locus for strains which stabilized Ycg1-3V5 (*pib1*Δ*, ydr306c*Δ*,* and *siz1*Δ). Strains were subjected to cycloheximide treatment, followed by western blot analysis of Ycg1-HA levels.



Appendix A.4: Ycg1 Stability is Not Regulated by the SCF. A) Cartoon of the Skp-Cullin-F-box E3 ligase complex (SCF). Cdc53 acts as a scaffold to structure the SCF and is necessary for complex function. B) A dominant-negative form of Cdc53 was expressed (*dnCDC53-GST*) under a galactose inducible promoter for 120minutes. C) The *cdc53-1* temperature sensitive mutant was inactivated by shifting cultures to 37C for 1hour prior to cycloheximide treatment. SCF cartoon adapted from Siergiejuk 2009.

Conclusions and Future Directions

The data presented in this appendix suggest that several genes associated with the UPS do not affect the turnover of Ycg1 upon deletion. In addition, SCF ligase function is not required for Ycg1 turnover. These data suggest that Ycg1 turnover; 1) was not adequately assessed in the screen, 2) requires one of the UPS modulating genes which was not included (gray rows), 3) is a target of an essential E3 ligase, or 4) is targeted by multiple E3 ligases.

In the future, UPS modulators which were not screened here should be deleted to determine their impact on Ycg1 turnover. Notably, one E3 ligase which was not included is MOT2, which has been shown to ubiquitinate chromatin-associated proteins, but is thought to require UBC4, which was deleted in the screen (Haworth 2010). The other E3 ligase that was not included is STE5, which contains a RING-H2 domain (Inouye 1997), is a putative ligase that has not yet been shown to ubiquitinate any target proteins.

In addition, essential E3 ligases should also be screened to determine their impact on Ycg1 turnover. Indeed, most of these have been shown to not modulate Ycg1 stability (data not shown), with the exception of RSP5 which has not been assessed. RSP5 is localized throughout the cell, and has been implicated in regulating gene expression, though is primarily thought to regulate intracellular trafficking (Kaida 2003).

If no single E3 ligase is shown to be necessary for Ycg1 turnover, then screening should test for interactions between each E3 ligase and Ycg1. This could be accomplished using a protein-fragment complementation assay such as yeast two hybrid or bimolecular complementation. In either case, the C-terminal region of *YCG1* would be fused a reporter gene fragment, while each E3 ligase would be fused to the complementary fragment of the reporter. If Ycg1 and an E3 ligase interact, the two fragments of the protein complement, which can be measured using by reporter activity, such as LacZ expression for the yeast two-hybrid assay. This type of analysis could identify an E3 ligase which interacts with Ycg1, but is not required for Ycg1 turnover; as is the case for redundant ligases (Landry 2012). Though labor intensive to establish, this screening method could be used to rapidly screen for interactions between other unstable proteins and the E3 ligase panel.

Methods

96-Well Yeast Transformation

Strains were grown in a deep well 96-well plate in YM-1 with 2% dextrose for two days. Cells were resuspended by vortexing, then 50uL was moved into a deep well 96-well plate with 100uL water to wash/remove media. 50uL of transformation mix (300mM Lithium Acetate, 16ug ssDNA, and 0.1ug plasmid per well) was added to each well, followed by 5minute vortexing at 300RPM. 100uL PEG 3350 was added at 50% v/w to each well, followed by 5min vortexing at 300RPM. The plate was then incubated at 42C for 1hour, followed by centrifugation and subsequent washing with water. The pellet was resuspended in 50uL water, and a 96-pin frogger was used to transfer a small amount of the suspension to a new 96-well plate with C-URA +2% dextrose media for selection.

Appendix A.5. Strains Table.

Name	Genotype	Background
YTD277	*MATa his3Δ leu2Δ met15Δ ura3Δ pRS306-YCG1p-YCG1-3V5*	*S288C*
YTD260	*MATa his3Δ leu2Δ met15Δ ura3Δ YCG1-3HA-HIS3 pib1::KanMx6*	*S288C*
YTD280	*MATa his3Δ leu2Δ met15Δ ura3Δ YCG1-3HA-HIS3 siz1::KanMx6*	*S288C*
YTD287	*MATa his3Δ leu2Δ met15Δ ura3Δ YCG1-3HA-HIS3 ydr307c::URA3*	*S288C*
YTD63	*MATa his3Δ leu2Δ met15Δ ura3Δ YCG1-3HA-HIS3 cdc53-1*	*S288C*

Appendix B: Interrupting Cyclical Ycg1 Expression via Constitutive Promoter Integration at the YCG1 Locus

Preface

Experiments in this appendix were designed and executed by Tyler Doughty.

Introduction

In chapter II, we show that cyclical expression of Ycg1 is a result of constitutive proteasomal turnover and cyclical transcription of *YCG1*. We report that *YCG1* transcription driven by the constitutive *TEF1* promoter interrupts cyclical expression, increases condensin levels, and delays G1/S progression. In this appendix, we explore other promoters which were assayed for constitutive expression of *YCG1*. The goal of these efforts was to constitutively express *YCG1* at 2 to 3-fold the wildtype protein level in asynchronous cells. We predict that this would result in increased condensin levels throughout the cell cycle, and similar proliferation defects to *TEF1p-YCG1*. If this is the case, then it might be beneficial to analyze the lower expression background in future works, since lower total expression decreases the chance of protein aggregation or other non-condensin related dysfunctions.

Results

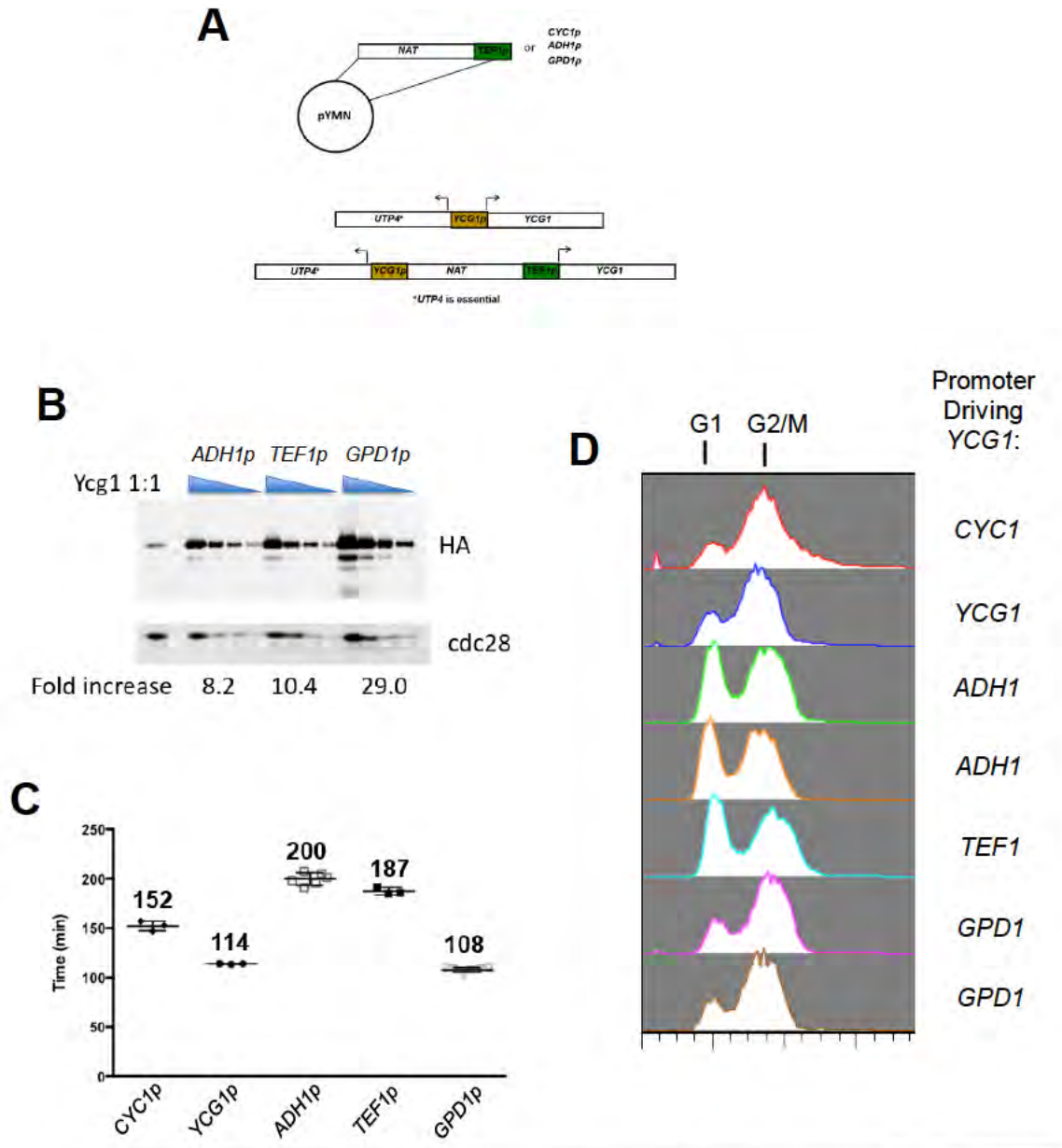
The top section of Appendix B.1.A shows the pYMN plasmid, which contains a *NAT* selection marker followed by a constitutive promoter (*TEF1*, *CYC1*, *ADH1*, or *GPD1*). Appendix B.1.A also shows the final product of *NAT-TEF1p* integration at the endogenous *YCG1* locus as an example. After integration, each promoter was assessed for Ycg1-HA expression levels via western blot (Appendix B.1.B). The *CYC1p-YCG1* strain was excluded from the analysis in B.1.B, since it's similar expression level allowed comparison to wildtype expression without dilution.

Altered *YCG1* transcription delays the cell cycle in the *CYC1*, *ADH1*, and *TEF1* promoter backgrounds (Appendix B.1.C). The *ADH1p-YCG1* stain exhibits slow proliferation and G1 accumulation, which mirrors *TEF1p-YCG1* phenotypes described in Chapter II (Appendix B.1.D and B.1.C respectively). Intriguingly, the *GPD1* promoter, which causes the highest level of Ycg1 expression, does not slow proliferation or cause G1 accumulation (Appendix B.1.C and B.1.D).

Though the *ADH1* promoter constitutively expresses *YCG1* at a lower level than *TEF1p*, while causing a similar phenotype, the overall expression in this background is still 8-fold increased compared to the wildtype. Thus, *ADH1p-YCG1* expression is still relatively high, and could lead to off-target consequences. To address these concerns, we attempted to constitutively express *YCG1* with a less drastic increase in protein level. Appendix B.2.A shows the restriction cloning

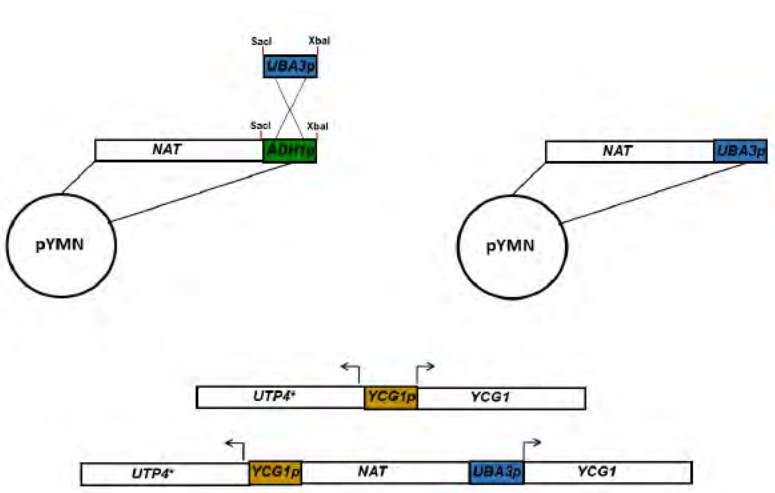
which allowed for replacement of the *ADH1* promoter on the pYMN plasmid backbone with any promoter of choice. This cloning method allows for integration of any new promoter, with NAT selection, and in the future is a viable method for rapid promoter replacement.

Appendix B.2.B shows the various promoters tested, and their observed expression levels. *LRO1p*, *SSL1p*, *UBA3p*, and *ENV10p* were inserted into the pYMN plasmid as described above and integrated into the genome. These promoters were chosen based on their respective gene's non-cyclical mRNA profiles. Non-cyclical transcription was predicted using cyclebase.org, a compilation of several yeast cell cycle transcription profiling datasets (Santos 2014). Protein level prediction is based on mass-spec data from Kulak 2014. At present, the concept of cloning and integrating these promoters has been demonstrated, but the effects on Ycg1 expression, cell cycle distribution, and doubling time has not been assessed.



Appendix B.1: Ycg1 Expression by Constitutive Promoter Integration. A) The pYMN templates encoding Nat selection followed by constitutive promoters of varying strength were integrated into the genome in front of *YCG1*. B) A dilution series of protein extracts from strains expressing Ycg1 under the indicated promoter. Note: dilutions of 1, 1:3, 1:6, and 1:12 are shown, fold increase was calculated by quantitating each dilution and creating a standard curve, which WT Ycg1 expression was fit to. Cdc28 is shown as a loading control, all expression of *YCG1* was from the endogenous locus. *CYC1-YCG1* levels were similar to wildtype (data not shown). C) Doubling times of strains with the indicated promoter driving *YCG1* transcription. Order is least expression (left) to highest expression (right). D) FACS plots for indicated promoter swaps.

A



B

Promoter	Protein molecules per cell*	Cyclebase.org rank**	Observed Ycg1 Expression
YCG1	103	394	1
TEF1	*630376*	*4681*	*10.4*
ADH1	*547967*	*4706*	*8.2*
GPD1	*24244*	*793*	*29.0*
CYC1	*1330*	*1610*	*0.3-1.1*
LRO1	**676**	**4997**	**-**
SSL1	**536**	**5982**	**-**
UBA3	**176**	**5986**	**-**
ENV10	**663**	**5993**	**-**

Appendix B.2: Ycg1 Expression by Novel Constitutive Promoter Integration. A) Various promoters were cloned into the pYMN template encoding Nat selection. Promoter insertion was confirmed by PCR, followed by amplification and integration at the YCG1 locus. B) A table showing novel promoters integrated at the YCG1 promoter locus **(*bold and italic*)** compared to the wildtype YCG1 promoter and previously described constitutive promoters *(italic)*. Observed expression relative to Ycg1 was calculated by quantifying western blot images and adjusting signal to wildtype YCG1. *Kulak 2014. **cyclebase.org - Santos 2014. Note, the yeast genome has 6,604 total open reading frames according to SGD.org.

Conclusions and Future Directions

From the data presented in Appendix B.1, we can conclude that expression of Ycg1 under the *ADH1* promoter causes slow proliferation and G1 accumulation similar to the *TEF1p-YCG1* background. Interestingly, the ADH1p-YCG1 background exhibits a minor, yet reproducible decrease in proliferation rate compared to *TEF1p-YCG1* (Appendix B.1.C and data not shown). In addition the *GPD1p-YCG1* background exhibited the highest total Ycg1 expression of the promoters tested, but did not impact the proliferation rate. One possible explanation for these observations is that the extreme elevation in Ycg1 expression in the *GPD1*-YCG1 background may cause feedback inhibition of other condensin subunits. Feedback inhibition may explain the ~40% reduction in Brn1-V5 levels in the *TEFp-YCG1-HA* overexpression background observed in Figure 2.12.A. In the future it will be interesting to probe for changes in Brn1 expression in the *GPD1-YCG1* background. In addition, integrating of *TEF-YCG1* into the *GPD1-YCG1* background would test the hypothesis that Ycg1 expression does not affect proliferation above a certain threshold.

Appendix B.1 also shows that *CYC1p-YCG1* causes slow proliferation. This phenotype may be due to decreased Ycg1 expression, which may reduce condensin levels during mitosis. A decrease in condensin levels could explain the presence of a G2/M shoulder in the FACS plots in Appendix B.1.D, as condensin has been shown to be necessary for mitotic progression (Freeman 2000).

In the future, the strategy described in Appendix B.2 might be useful to achieve constitutive Ycg1 expression at closer to wildtype levels in asynchronous cells. However, *LRO1p*, *SSL1p*, *UBA3p*, and *ENV10p* should be assessed first, since their expression of Ycg1 could inform future promoter replacement strategies (Appendix B.2.B). Nonetheless, constitutive Ycg1 expression at similar to wildtype levels should be pursued to ensure that constitutive expression, and not overexpression, causes the observed phenotypes in the *TEF1p-YCG1* background.

Methods

Promoter Integration

Appendix B.1.A shows the strategy for integrating exogenous promoters at the *YCG1* locus. Briefly, PCR primers containing homology to the upstream region of *YCG1* were used to amplify a segment of the pYMN plasmid which contains a NAT resistance gene, followed by the promoters listed in B.1.A. Strains were transformed with the PCR products above and selected under nourseothricin drug selection, followed by confirmation of promoter integration by colony PCR (Janke 2004).

Quantitative Western Blotting and Analysis

Western blots were imaged on a BioRad ChemiDoc imager and quantitation was carried out using BioRad ImageLab software. Signal from each band in the HA blot was normalized to its corresponding cdc28 loading signal. Ycg1 expression level was determined by creating a standard curve of expression signal versus dilution for each promoter and fitting wildtype Ycg1 to the curve (Appendix B.1.B).

Rapid Integration of Novel Promoters into the pYMN Plasmid Backbone

Novel promoters were cloned into the pYMN plasmid backbone using restriction digested PCR amplified promoters. This was accomplished by amplifying the upstream promoter sequence with a 29-mer which includes 5’

cgaGAGCTC followed by upstream promoter homology. Here, cga is excess to aid in restriction digestion (cutaway sequence), and GAGCTC is a SacI restriction digestion site. The reverse PCR primer was created by adding cgaTCTAGA to the 5'end of the reverse compliment of the 3' promoter region. The final PCR product is cutaway-SacI-5'-promoter-3'-XbaI-cutaway. This PCR product was digested with SacI and XbaI and inserted in the place of the *ADH1p* in the pYMN-*ADH1p* plasmid. Notably, promoters with internal XbaI and SacI digestion sites are not candidates for insertion via this method, though other restriction digestion sites might be used in these cases.

Appendix B.3. Strains Table.

Name	Genotype	Background
YTD33	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 YCG1-3HA-HIS3*	W303
YTD336	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TEF1p-YCG1-3HA-HIS3*	W303
YTD404	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-CYC1p-YCG1-3HA-HIS3*	W303
YTD405	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-ADH1p-YCG1-3HA-HIS3*	W303
YTD406	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-GPD1p-YCG1-3HA-HIS3*	W303

Appendix C: Condensin Subunit Fusion to an Exogenous Degron Rescues *ycg1-K977A* Proliferation

Preface

Experiments in this section were designed and executed by Tyler Doughty.

Introduction

A degron is typically a small stretch of a protein that negatively influences it's stability, often by direct interaction with an E3-ligase (Hochstrasser 1996, Ravid 2008). In Chapter II, we present the Ycg1 Lysine-977 to Alanine mutation, which likely interferes with degron function and stabilizes Ycg1. Since the E3 ligase recruitment by many degrons is transferable (Ravid 2008), we hypothesized that fusion of an exogenous degron to Ycg1-K977A would rescue its instability and might therefore rescue proliferation defects caused by increased expression. In addition, we hypothesized that fusing a degron to another condensin subunit might reduce that subunit to rate limiting levels, and thus rescue the constitutive Ycg1 expression phenotypes. The goal of these efforts was to target condensin subunit destruction with a degron which is actively recognized by a nuclear E3-ligase after chromosome segregation.

Two candidate degrons from the proteins Cin8 and Cdc20 were chosen for condensin subunit fusion (Hildebrandt 2001, Arnold 2014). Cin8 and Cdc20 are

targets of the APC^{Cdh1}, which targets nuclear proteins for turnover from late mitosis until late G1 (Hildebrandt 2001, Morgan 2007, Arnold 2014). During the remainder of the cell cycle, Cdh1 is held in an inactive state due to Cdk phosphorylation, which prevents it from targeting proteins for turnover (Morgan 2007). This cell cycle dependent activation and targeting made Cdh1 an attractive candidate for inducing a G1 drop in condensin subunit levels in the constitutive Ycg1 expression backgrounds. We predict that this decrease in Ycg1 would decrease total condensin formation and rescue the proliferation defects described in Chapter II.

Results

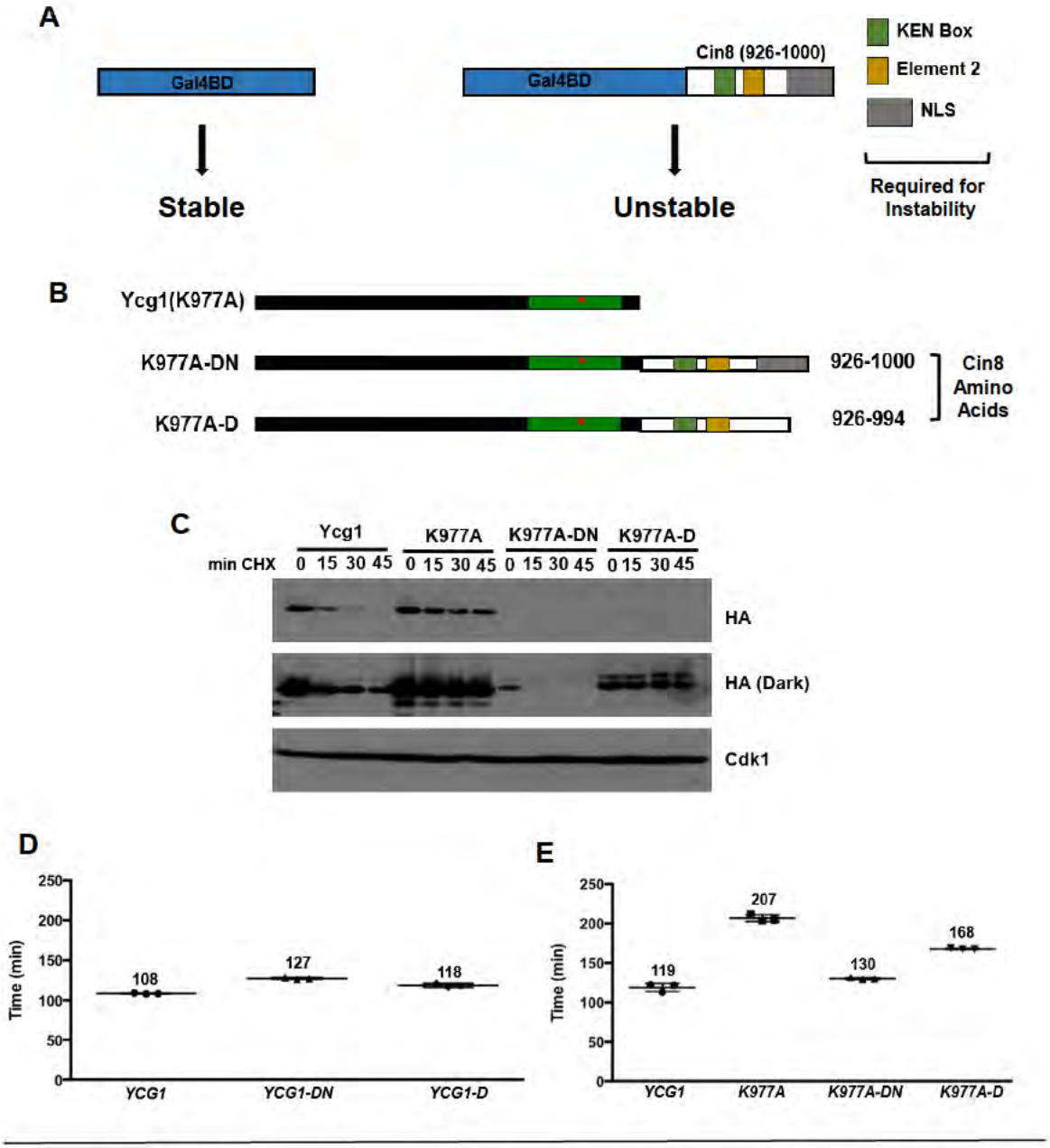
Degron Fusion Chimeras Partially Rescue Proliferation Defects Associated with *ycg1-K977A*.

Appendix C.1.A shows a cartoon summary of a key experiment which found that the Cin8 C-terminus is sufficient to induce degradation of the otherwise stable Gal4-binding domain in G1 arrested strains (Gal4BD) (Hildebrandt 2001). Further analysis showed that APC^{Cdh1} function is necessary for Cin8 and Gal4BD turnover (Hildebrandt 2001). Notably, the work which characterizes Cin8 suggests that the protein is 1031 amino acids long, while the *Saccharomyces* Genome Database lists this protein as 1000 amino acids. Nonetheless, the C-terminal amino acids described below are common between both the sequences in SGD and Hildebrandt et. al 2001. Coordinates from SGD are shown (Appendix C.1.A).

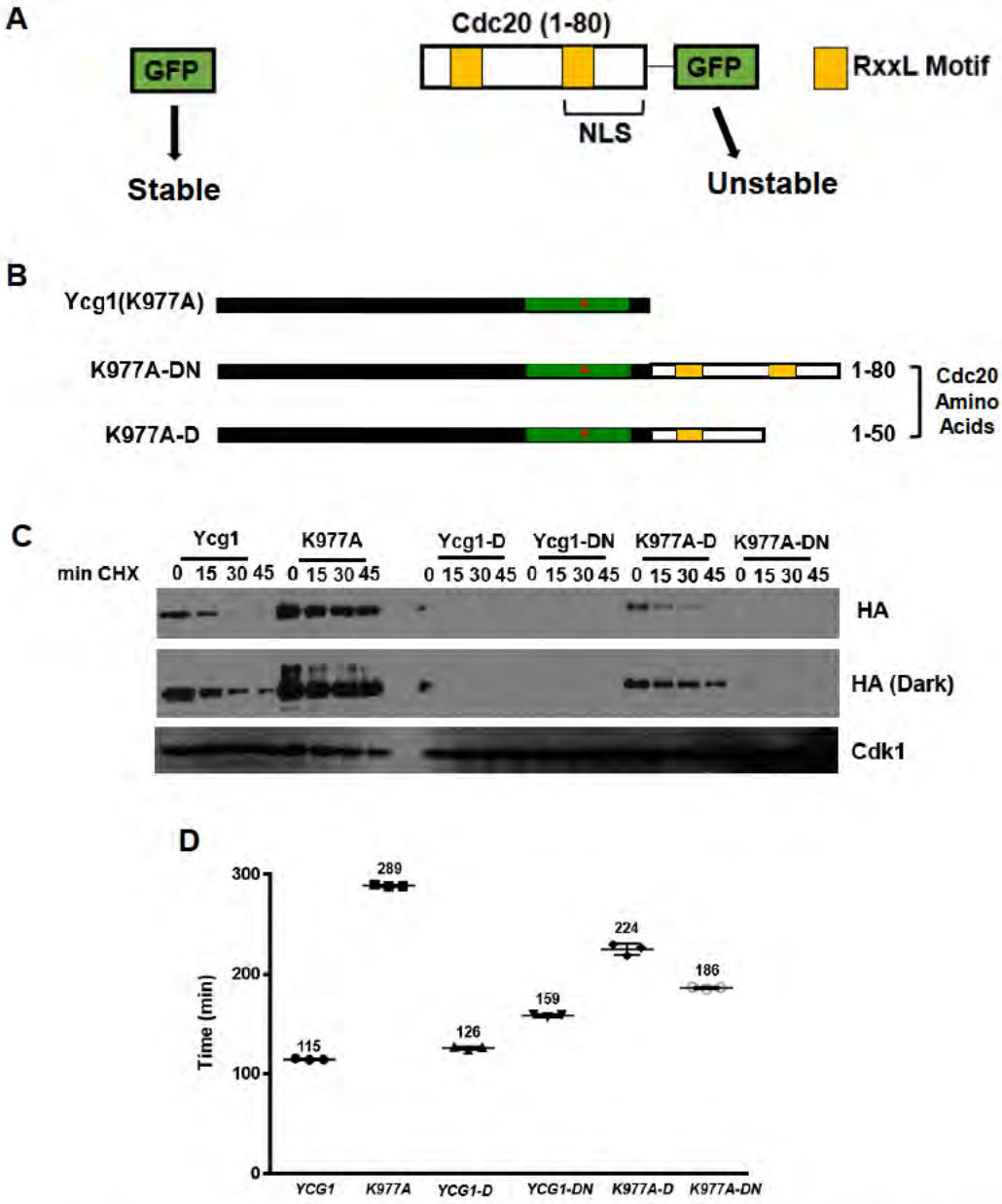
Proteasomal targeting of Cin8 requires two C-terminal elements of stability and a nuclear localization signal (NLS) (Appendix C.1.A). The *CIN8* degron-NLS region or the degron region alone was fused to *YCG1* and *ycg1-K977A* (Appendix C.1.B). Stability was assessed via cycloheximide-chase assay in asynchronous strains, which showed that Ycg1-K977A levels were reduced after fusion of the Cin8 degron, and instability was restored upon fusion of the Cin8 degron-NLS (Appendix C.1.C). Notably, *CIN8* degron fusion to *ycg1-K977A* partially rescued proliferation, while *CIN8* degron/NLS fusion rescued *ycg1-K977A* strains to near wildtype proliferation. However, the complete turnover of these fusion proteins in asynchronous strains was unexpected, since Cin8 degron activity is restricted to late mitosis and early G1 (Hildebrandt 2001). Notably, published data shows that Cin8 is relatively stable in asynchronous cultures (Hildebrandt 2001), likely since only a fraction of the asynchronous population has active APC^{Cdh1}.

The possibility that the Cin8 degron region might destabilize Ycg1 throughout the cell cycle, and not just during G1, inspired us to attempt to destabilize Ycg1 with a different degron. The degron we chose is from the Cdc20 protein, which, like Cin8, is targeted for destruction by APC^{Cdh1} during late mitosis/early G1. Appendix C.2.A summarizes data from Arnold et al. 2014, which showed that Cdc20's N-terminal 50 amino acids constitute a Cdh1 targeted degron, while AA51-80 act as a nuclear localization signal. *YCG1* and *ycg1-K977A* were fused to the N-terminal regions of *CDC20* which code for the degron or degron-NLS (Appendix C.2.B). Once again, these fusions resulted in undetectable

levels of Ycg1 and destabilized *ycg1-K977A* in asynchronous strains (Appendix C.2.C). Together, these experiments suggest that Ycg1 has reduced stability upon fusion to either the Cin8 or Cdc20 degron, and further reduced stability upon fusion to either degron-NLS (Appendix C.1.C and C.2.C). However, in all cases, turnover of these constructs does not appear to be dependent on APC^{Cdh1} function during G1.



Appendix C.1: Rescuing the Instability of ycg1(K977A) with Cin8 Degron-NLS Construct. A) A cartoon summarizing the Cin8 degron and NLS characterization in Hildebrandt 2001. Cin8 turnover depends on it's KEN box, Element 2, and its nuclear localization signal, which depends on the C-terminal 30 amino acids. B) Cin8 degron and degron-NLS were fused to Ycg1-K977A. C) Cycloheximide stability assay in asynchronous strains shows that Ycg1-K977A-HA (lower band) instability is rescued by addition of the Cin8 degron and NLS, but not the degron alone. D) and E) Cin8 degron fusion partially rescues growth, while Cin8 degron/NLS strongly rescues growth in the *ycg1-K977A* background. Technical replicates from a single experiment are shown.

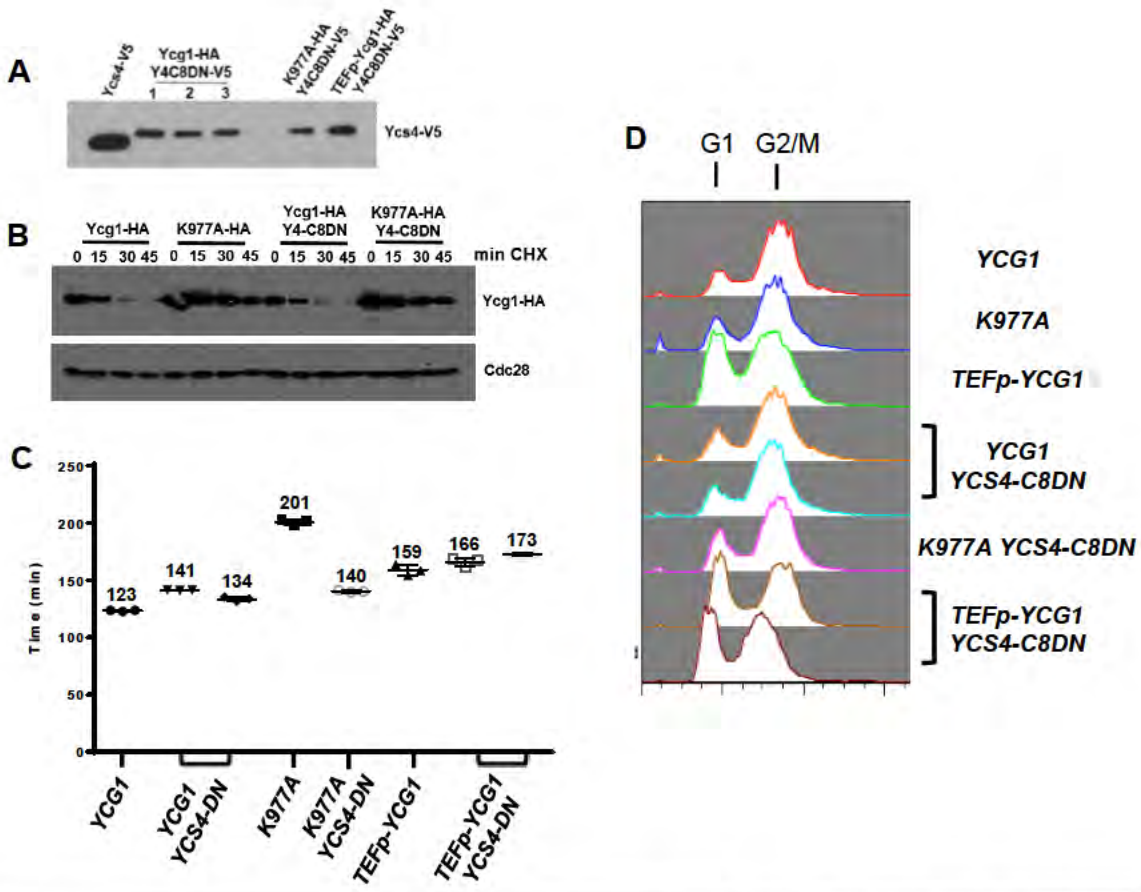


Appendix C.2: Rescuing the Instability of Ycg1-K977A with Cdc20 Degron-NLS Construct. A) A cartoon summarizing the Cdc20 degron and NLS characterization in Arnold 2014. Cdc20 turnover depends on it's RxxL motifs, one of which includes an NLS sequence. B) The Cdc20 degron (D) or Cdc20 degron-NLS (DN) were fused to *YCG1* or *ycg1-K977A*. C) Cycloheximide stability assay in asynchronous strains shows that Ycg1-K977A-HA instability is rescued by addition of the Cdc20 degron or degron-NLS. D) Cdc20 degron-NLS fusion slows proliferation in the wildtype *YCG1* background, and partially rescues growth in the *ycg1-K977A* degron only and degron-NLS backgrounds. Technical replicates from a single experiment are shown.

Ycs4 Degron Fusion Rescues Proliferation Defects Caused by *ycg1-K977A*

Since increased Ycg1 expression delays the progression through the early cell cycle and increases condensin formation, we concluded that Ycg1 is the rate limiting subunit for condensin formation (Chapter II). The concept of a rate limiting subunit relies on the observation that all subunits of condensin are essential for viability and measurable condensin function (Lavoie 2002, Lavoie 2004, D'Ambrosio 2008). Therefore, we hypothesized that decreasing another condensin subunit to limiting levels might rescue the phenotypes associated with increased Ycg1 expression.

The *YCS4* subunit of condensin was fused to the aforementioned *CIN8* C-terminal region, which codes for a degron and a nuclear localization signal, shown in Appendix C.1.A. *CIN8* degron/NLS fusion to *YCS4* led to decreased Ycs4 protein levels in asynchronous cells in the *YCG1-HA*, *ycg1-K977A*, and *TEFp-YCG1* strain backgrounds (Appendix C.3.A). However, this difference in asynchronous protein was modest compared to Ycg1-Cin8DN fusions (Appendix C.1.C). Ycg1 and Ycg1-K977A turnover was unaffected in the *YCS4-cin8DN* background (Appendix C.3.B), and *YCS4-cin8DN* rescued the proliferation defect of the *ycg1-K977A* strain, but did not improve the proliferation rate of *TEFp-YCG1* strains (Appendix C.3.C).



Appendix C.3: Decreasing Ycs4 Expression via Cin8 Degron Fusion. A) The Cin8 Degron/NLS construct from C.1.A was fused onto *YCS4* at its endogenous locus. Total Ycs4-V5 in asynchronous cells was monitored via western blot. B) Ycg1-HA expression over time in the presence of cycloheximide is shown, in the presence or absence of Ycs4-Cin8DN-V5. C) Growth rates for *YCG1*, *ycg1-K977A*, and *TEFp-YCG1* are shown alongside their respective *YCS4-C8DN* counterparts. Technical replicates from a single experiment are shown. D) Asynchronous FACS profiles of strains shown in C.4.C.

Conclusions and Future Directions

Together, the Ycg1 degron fusion chimeras suggest that Ycg1-K977A interrupts a degron, and that rescuing Ycg1 instability partially rescues proliferation. Unexpectedly, the instability observed appears not to depend on APC^{Cdh1}, which acts specifically during late mitosis and early G1 to target Cin8 and Cdc20. Instead, turnover likely occurs throughout the cell cycle, as asynchronous degron fusion strains show complete Ycg1 and Ycg1-K977A turnover (Appendix C.1.C and C.2.C). These data suggest that Ycg1 instability might result from increasing the length of its C-terminal tail. Notably, C-terminal tags used in Chapter II, including the 238AA GFP tag, do not appear to decrease the half-life of Ycg1 compared to N-terminal tags (data not shown). Thus, instability may be caused by the addition of an unstructured and/or a basic region, both of which are common amongst degrons and NLSs.

Although unstable throughout the cell cycle, these strains could be used to determine whether restoring Ycg1-K977A instability decreases condensin formation, as we might predict from data presented earlier (Figure 2.11). In the future, this could be tested via Co-IP of condensin subunits during cell cycle arrests. In particular, it might be interesting to compare the interaction between condensin subunits and Ycg1, Ycg1-K977A, or Ycg1-K977A-Cin8DN during early interphase. If Ycg1 is limiting for condensin formation, we expect that Ycg1-K977A-Cin8DN, which is less expressed than wildtype Ycg1, will cause lower than wildtype condensin formation.

Ycg1-Cin8 degron and degron-NLS protein stability data presented in Appendix C.1.C suggest a possible role for the Cin8 NLS in destabilizing Ycg1-K977A. These data are somewhat supported by Cdc20 degron-NLS fusion, which exhibits lower expression then the Cdc20 degron alone (Appendix C.2.C). Furthermore, proliferation was rescued more strongly in both degron-NLS fusions compared to degron fusions alone (Appendix C.1.D-E and C.2.D). However, these fusions did not turnover Ycg1 as expected, and instability may be due to non-specific mechanisms that are exacerbated by the additional size or basic amino acid composition of the NLS regions. Alternatively, it is possible that NLS function is influencing Ycg1 turnover by altering its nuclear localization. In the future, these degron and degron-NLS fusion constructs should be fused to the N-terminus of Ycg1 to evaluate their effects on proliferation.

YCS4 fusion to *CIN8* degron-NLS constructs resulted in a more modest drop in protein expression as compared to *YCG1-cin8DN* (Appendix C.1.C t=0 and C.3.A). This may be due to Ycs4-Cin8DN targeting by APC^{Cdh1} during late mitosis and early G1, which represents a fraction of the total asynchronous culture. Future experiments should test the half-life of Ycs4-Cin8DN in asynchronous and G1 arrested strains. If turnover is restricted to G1, then the *YCS4-cin8DN* construct may prove to be ideal for testing the rate-limiting subunit model and condensin's effect on G1/S progression.

Additionally, if Ycs4-Cin8DN instability is APC^{Cdh1} specific in this background, then the growth data in Appendix C.3.C will be of particular interest

to confirm. Specifically, it will be interesting to determine how the *YCS4* degron fusion rescues the *ycg1-K977A* proliferation defect, but not the *TEFp-YCG1* proliferation defect. This difference might help further elucidate how these two perturbations affect proliferation. If condensin formation in early interphase is decreased upon Ycs4 turnover in the *TEFp-YCG1* background, then the *TEFp-YCG1* proliferation phenotype may be independent of condensin function. Conversely, if condensin formation is unaffected by Ycs4 turnover, then the rescue of *ycg1-K977A* may be a result of restoring a function of the Ycg1 protein, such as subcellular or chromatin localization.

Methods

CIN8 and *CDC20* Degron Fusion to Condensin Subunits

Both *CIN8* and *CDC20* degron and degron/NLS regions were fused to the *YCG1* or *YCS4* C-termini at their endogenous loci in a haploid yeast. Integration was achieved via homologous recombination with 50 base pair overhangs on each side, flanking the stop codon of the condensin subunit of interest. All insertions were isolated by marker selection, followed by colony PCR. Each *CIN8* and *CDC20* truncation (described below) was confirmed by sequencing.

DNA coding for the *cin8D* (AA926-994) and *cin8DN* (AA926-1000) regions, plus an epitope tag and a selection marker were amplified directly from genomic DNA for integration downstream of condensin subunit genes. Since the *cin8DN* region resides at the 3' end of *CIN8*, it was amplified directly from full length *CIN8-HA-HIS3* strains. In order to amplify the degron only, the final seven C-terminal codons were removed (including the stop codon), and replaced with a tagging cassette, yielding *cin8(1-2982)-HA-HIS3*. *CIN8* is not an essential gene, so all genetic manipulation was done at the endogenous locus in haploid yeast.

CDC20 is essential, and it's degron resides at the 5' end of the gene. In order to create a template for degron and degron/NLS fusion, one copy of the *CDC20* gene was truncated leaving either 240 or 150 basepairs of the 5' end of the gene intact in diploid yeast. This yielded a *cdc20(1-240)-HA-HIS3/CDC20* and a *cdc20(1-150)-HA-HIS3/CDC20* diploid strain. Genomic DNA from these strains

were was subsequently used to amplify the *cdc20D-HA-HIS3* and *cdc20DN-HA-HIS3* constructs for integration downstream of *YCG1*.

Appendix C.4. Strains Table.

YTD33	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 YCG1-3HA-HIS3*	W303
YTD148	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-K977A-3HA-HIS3*	W303
YTD315	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-cin8(926-1000)-3HA-HIS3*	W303
YTD316	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-K977A-cin8(926-1000)-3HA-HIS3*	W303
YTD323	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-cin8(926-994)-3HA-HIS3*	W303
YTD325	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-K977A-cin8(926-994)-3HA-HIS3*	W303
YTD338	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-cdc20(1-50)-3HA-HIS3*	W303
YTD339	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-cdc20(1-80)-3HA-HIS3*	W303
YTD340	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-K977A-cdc20(1-50)-3HA-HIS3*	W303
YTD341	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-K977A-cdc20(1-80)-3HA-HIS3*	W303
YTD61	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 YCS4-3V5-kanMX6*	W303
YTD372	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 YCG1-3HA-HIS3 ycs4-cin8(926-1000)-3V5-kanMX6*	W303
YTD373	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ycg1-K977A-3HA-HIS3 ycs4-cin8(926-1000)-3V5-kanMX6*	W303
YTD374	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Hyg-TEF1p-YCG1-3HA-HIS3 ycs4-cin8(926-1000)-3V5-kanMX6*	W303

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